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Cortical Cannabinoid Modulation of Subcortical Dopamine Activity: Implications for Emotional Processing

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Graduate Program in Neuroscience
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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CORTICAL CANNABINOID MODULATION OF SUBCORTICAL
DOPAMINE ACTIVITY: IMPLICATIONS
FOR EMOTIONAL PROCESSING

by

Brittany Draycott

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science

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London, Ontario, Canada

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ABSTRACT

Humans receive countless sensory inputs from the outside world to which they assign a certain level of emotional significance. However, there are times when an individual may assign an abnormally high level of emotional salience to an otherwise non-significant event, resulting in an inappropriate allocation of attention as seen in the hallucinations and psychosis associated with schizophrenia. Several brain regions are involved in this emotional processing, including the medial prefrontal cortex (mPFC) and the ventral tegmental area (VTA). We have previously shown that activation of mPFC cannabinoid (CB1) receptors in rats causes a potentiated fear response to a normally non-salient sensory event (Laviolette & Grace, 2006a). To further investigate the cause of this increased fear response we performed *in vivo* extracellular electrophysiology recordings along with olfactory fear conditioning in awake, behaving animals. We observed a biphasic effect across both methodologies where an intra-mPFC low dose CB1 agonist caused an increase in spontaneous neural activity in VTA DA cells and a potentiated fear response, while an intra-mPFC high dose CB1 agonist caused a decrease in spontaneous neural activity in VTA DA cells and a block of normal fear memory acquisition, an effect that was rescued using an intra-VTA GABA-B receptor antagonist. Given the implications of the dopamine and cannabinoid systems in schizophrenia, these studies could provide important insights into the underlying neural activity of the emotional processing deficits associated with the disorder.

Keywords: Prefrontal Cortex, Ventral Tegmental Area, Cannabinoids, Dopamine, Electrophysiology, Fear Conditioning, GABA, Emotion Processing

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
α -flu	α -flupenthixol
AM-251	CB1 receptor antagonist
BLA	basolateral nucleus of the amygdala
CB1	cannabinoid CB1 receptor
CNS	central nervous system
CPP	conditioned place preference
DA	dopamine
GABA	gamma-aminobutyric acid
ICSS	intra-cranial self stimulation
IL	infralimbic division of the prefrontal cortex
i.p.	intra-peritoneal
i.v.	intra-venous
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
PFC	prefrontal cortex
PLC	prelimbic division of the prefrontal cortex
PTSD	post-traumatic stress disorder
THC	tetrahydrocannabinol
VTA	ventral tegmental area
WIN55	CB1 receptor agonist

1. Introduction

Under normal circumstances, people receive countless sensory inputs from the outside world to which they assign appropriate emotional significance. Through this mechanism, we are able to react accordingly based on the emotional importance or non-importance of an event. However, there are times when this emotional processing mechanism does not work as it should, as in schizophrenia or post-traumatic stress disorder (PTSD). An individual may assign an abnormally high level of emotional salience to an otherwise non-significant event resulting in an inappropriate allocation of attention and, in extreme cases, hallucinations and psychosis. Thus far, a considerable body of research has identified several brain regions involved in this emotional processing circuit, including the medial prefrontal cortex (mPFC) and the ventral tegmental area (VTA). These areas comprise components of the mesocorticolimbic circuit, and contain high levels of dopamine (DA) and cannabinoid CB1 receptors, which both play a critical role in modulating emotionally salient stimuli. Anatomically, the mPFC and VTA communicate via ascending and descending functional projections and can bi-directionally modulate neuronal activity dynamics across the pathway. Additionally, the DA and CB1 systems have been shown to functionally interact with each other and are implicated in the etiology and treatment of schizophrenia. In this thesis, I will investigate the interaction between these neurotransmitter systems within the mesocorticolimbic circuit, a neural system critical for emotional processing. Specifically, my research focuses on how cortical cannabinoid transmission can alter sub-cortical neuronal activity patterns in the VTA and thereby modulate the salience of incoming sensory information and associative memory formation. In addition, I will investigate the

specific pharmacological mechanisms controlling functional interactions between the PFC and VTA, in the context of emotional processing, at the neuronal and behavioural levels of analysis.

1.1 Emotional processing and the mesocorticolimbic system

1.1a The ventral tegmental area

The mesocorticolimbic system is often known as the “reward” circuit in the brain as it’s the system that mediates the rewarding properties of various commonly abused drugs like cocaine, morphine, nicotine and amphetamine (Wise, 2004). However, this circuit also plays an important role in emotional processing in a more general sense, including the processing of other emotionally salient experiences, such as aversive stimuli. Situated deep within the midbrain is the VTA. The VTA contains a large number of DA cell bodies, classified as the “A10” neurons, that project to different structures within the mesocorticolimbic circuit, including the amygdala, the nucleus accumbens (NAc) and the prefrontal cortex (PFC). This dopamine signal has been shown to play a number of different functional roles including the processing and integration of emotionally salient information (Laviolette & Grace, 2006b), which will be discussed later in more detail. While each area of the mesocorticolimbic circuit plays an important role, the functional and anatomical connections between the VTA and the mPFC have been extensively studied and are the main focus of pharmacological manipulation in this thesis.

The direct and reciprocal neural projections from the VTA to the mPFC have been characterized previously. Using a triple-labelling procedure that combined anterograde and retrograde tracing with immunocytochemical identification, Sesack and Carr (2002) were able to show that glutamatergic projections from the mPFC innervated only the DA cells in the VTA that project back up to mPFC, and not DA cells that project to different parts of the circuit such as the NAc. This suggests evidence of a closed-circuit feedback loop, where the activity of mesocortical DA cells directly influence cortical activity in mPFC and vice versa. Indeed, Peters, Lewis and O'Donnell (2000) demonstrated the synchronous activity between the VTA and mPFC using intra- and extracellular recordings and showed that DA activity in the VTA was able to control membrane potential states of pyramidal cells in mPFC. But the effect is not just limited to pyramidal cells – Tseng et al. (2006) were able to show that VTA stimulation directly altered the firing frequency of fast-spiking interneurons located in the mPFC. Conversely, stimulation of neurons in the PFC have been shown to have both excitatory and inhibitory effects on DA cells in the VTA (Gao et al., 2007). Thus, the anatomical connections between these two areas are well-known but what does this mean on a functional level? The functions of the mesolimbic pathway are numerous but have been shown to be important in the processing of emotionally salient information. Acting mainly through a DA-dependent mechanism, the VTA-PFC pathway can encode signals of both salience and valence, which will be discussed in more detail below (see 1.1c).

1.1b The prefrontal cortex

The PFC plays a crucial role in mediating various behaviours, allocating attention, working memory, decision-making and regulating subcortical brain structures, among other things. As discussed previously, it receives projections from the VTA along with most other brain structures including those in the mesocorticolimbic circuit such as the NAc and basolateral amygdala (BLA). It also sends afferent fibres to many of those areas in return, providing a modulatory “top-down” effect on a wide range of neural activity. The PFC is a relatively large region and can be subdivided into different structural areas with unique, yet overlapping, functions. The mPFC includes both the infralimbic (IL) and prelimbic (PL) cortices and has been implicated in the processing of associative memories, including the acquisition, maintenance and extinction phases (Miller, 2000). In fact, research has shown the ability of the mPFC to encode emotional conditioned associations exists at both the behavioural and the single neuron level. Using an olfactory fear conditioning procedure, Laviolette, Lipski and Grace (2005) demonstrated that individual neurons in the mPFC were able to show a learned association to an emotionally salient associative fear stimulus. In contrast, neurons in the more ventral IL, show increased associative activity during the extinction (unlearning) of previously acquired associative fear memories (Milad & Quirk, 2002). Furthermore, Milad, Vidal-Gonzalez and Quirk (2004) were able to show this effect at a behavioural level as well, reporting that stimulation of the IL could modulate the expression of a conditioned fear response. These reports are consistent with various other studies implicating the mPFC as an important brain region for emotional memory processing (Feenstra, Vogel, Botterblom, Joosten, & Bruin, 2001; LeDoux, 1995; Miller, 2000; Williams et al., 2004).

The functional role of the mPFC in emotional processing becomes increasingly evident when examining disorders that are characterized by structural and functional mPFC deficits, such as schizophrenia (Laviolette & Grace, 2006b). Schizophrenia is a complex, multifaceted neuropsychiatric disorder exhibiting a plethora of symptom profiles. However, there are various aspects of the disease that have been repeatedly observed, one of which includes prefrontal abnormalities. Brain volume reduction has been shown, as well as reduced connectivity from the PFC to other brain regions (Keshavan, Tandon, Boutros, & Nasrallah, 2008). Also, tasks known to involve frontal lobe function are often performed poorly by patients with schizophrenia (Berman et al., 1997; Keshavan et al., 2008). Williams et al. (2004) conducted an imaging study to examine how schizophrenia patients processed images of fearful stimuli versus controls. They examined brain activity as well as skin conductance and found that paranoid schizophrenic patients displayed heightened autonomic response but *reduced* neural activity in the prefrontal-amygdala circuit. This not only shows reduced activity in the PFC but also an interesting disconnect between the autonomic response to an emotional cue and the corresponding neural activity.

1.1c Dopamine and emotional processing

As discussed previously, both the VTA and mPFC have been implicated for their role in the processing of emotionally salient stimuli. Much of their involvement can be attributed to the activity of DA throughout the mesocorticolimbic system, where a large number of DA receptors are concentrated. Thus far, two main families of DA receptors have been classified. They are divided into D1-like and D2-like subtypes, and have

differing synaptic locations and intracellular signalling effects. The D1-like subtype consists of the D1 and D5 receptor types, which are mainly found post-synaptically and whose activation generally stimulates adenylyl cyclase. In contrast, the D2-like subtype consists of the D2, D3 and D4 receptors which can be located both pre- and post-synaptically and whose activation generally inhibits adenylyl cyclase (Missale, Nash, Robinson, Jaber, & Caron, 1998; Vallone, Picetti, & Borrelli, 2000).

A large cluster of DA cell bodies resides in the VTA and it is from here that they have their well-known effect on drug addiction and reward. Through projections to the BLA, NAc and PFC, DA activity has also been shown to play an important role in how the brain processes the salience of specific sensory stimuli (Adinoff, 2004; Berridge & Robinson, 1998). For example, Nader & LeDoux (1999) found that systemic application of quinpirole activated DA autoreceptors, leading to a decrease in DA transmission that correlated with a decrease in fear behaviour by blocking the retrieval of a learned CS-US association. In a similar vein, neuroleptic medications that act through antagonizing DA receptors (specifically clozapine and haloperidol) were found to block the acquisition of a conditioned fear association (Inoue, Tsuchiya & Koyama, 1996). Conversely, there are a number of studies showing the opposite effect – an increase in DA activity in the mesocorticolimbic circuit can lead to potentiated fear responses and heightened conditioned fear associations (Killcross, Everitt, & Robins, 1997; Morrow, Elsworth, & Roth, 1996; Miczek & Luttinger, 1978; Borowski & Kokkinidis, 1998).

Detailed work has been conducted looking at the role of DA in the mPFC specifically, and how dopaminergic transmission in this brain region can affect emotional processing. Shah, Sjovold and Treit (2004) examined the anxiolytic effects of blocking

DA receptors in the mPFC during elevated plus maze and shock-probe burying tests. They found that antagonizing mPFC D4 receptors led to a decrease in fear-related behaviour in both tests, demonstrating an important role for the D4 receptor in processing emotionally salient information. Similar results have been found using an olfactory fear conditioning paradigm, where systemic administration of a D4 antagonist was able to block the ability of single mPFC neurons to learn an olfactory fear association. This effect was replicated at the behavioural level, where mPFC infusions of the same D4 antagonist were able to block the expression of a normal fear response to a footshock-paired odor (Laviolette et al., 2005). Further investigation revealed a bi-directional role for D4 receptors during behavioural fear conditioning, where activation of mPFC D4 receptors was able to potentiate a fear response to a normally non-salient subthreshold footshock while also blocking a normal fear response to a suprathreshold footshock (Lauzon, Bishop, & Laviolette, 2009). Yet DA modulation of emotional processing is not limited to the D4 receptor subtype. For example, the D1 subtype has also been shown as a crucial mediator in the processing of emotionally salient stimuli. Activating D1 receptors in mPFC can block the expression of a suprathreshold associative fear memory that has been learned previously (Lauzon et al., 2009) and can also block the expression of a previously learned morphine reward memory, an effect that can be rescued by co-administration of a cAMP signaling inhibitor (Lauzon, Bechard, Ahmad, & Laviolette, 2013).

DA dysfunction is associated with a number of different disorders ranging from Parkinson's disease to Tourette's syndrome to schizophrenia (Missale et al., 1998). The role of DA dysfunction in schizophrenia will be discussed in further detail below and

while its importance is evident, DA is not the sole neurotransmitter involved in emotional processing. Another system, the cannabinoid system, is also crucial for signalling the importance of emotional stimuli.

1.2 The cannabinoid system

1.2a Actions in the central nervous system

Two main cannabinoid receptors, CB1 and CB2, have been characterized thus far. As CB2 has been found predominantly in the periphery, we focused our attention specifically on the CB1 receptor. Within the central nervous system (CNS) the CB1 receptor is prominent and can be found in areas associated with emotional processing such as the amygdala, striatum, hippocampus, and frontal cortex (Dove Pettit, Harrison, Olson, Spencer & Cabral, 1998; Moldrich & Wenger, 2000). CB1 is an inhibitory G-protein coupled receptor and is located presynaptically, where it inhibits the release of neurotransmitters by a number of different signalling events including the closing of Ca^{2+} channels, opening of K^{+} channels, and the inhibition of adenylyl cyclase activity (Hirvonen et al., 2012; Lupica, Riegel, & Hoffman, 2004; Piomelli, 2003). CB1 receptors have been found in areas of both the mPFC and the VTA (Moldrich & Wenger, 2000; Tsou, Brown, Sañudo-Peña, Mackie, & Walker, 1998; Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006) which, as discussed previously, provide important modulation for the processing of emotionally salient information.

1.2b Cannabinoids and emotional processing

Several lines of evidence exist showing the importance of CB1 receptor activity in emotion-related behaviours and the processing of emotionally relevant stimuli. In human self-report studies, cannabinoid consumption was shown to cause mood-related disturbances and influence sensory perception in participants, along with altering the significance of incoming sensory information (Green, Kavanagh, & Young, 2003; Wachtel, ElSohly, Ross, Ambre, & de Wit, 2002). In animal studies, Marsicano et al. (2002) report that CB1 knockout mice are unable to extinguish an aversive conditioned fear memory of an auditory cue paired with a footshock. CB1 knockout mice have also been shown to display abnormal emotional responses to behavioural paradigms like the resident-intruder test and a chronic unpredictable mild stress paradigm (Martin, Ledent, Parmentier, Maldonado, & Valverde, 2002). Behaviours such as increased aggression and heightened depressive responses were commonly observed, implying that the CB1 receptor is essential for normal emotional behaviours. Pharmacological modulation of CB1 receptors has also been shown to affect the salience of incoming sensory information. Laviolette and Grace (2006a) performed olfactory fear conditioning experiments and demonstrated an increased fear response to a normally non-fearful stimulus in rats during mPFC CB1 activation, an effect that was completely blocked using a CB1 antagonist. Furthermore, CB1 receptor transmission along the BLA-mPFC pathway appears to be crucial for the encoding of emotional memories, with both systemic and local infusions of the CB1 antagonist AM-251 blocking the acquisition of a conditioned fear association (Tan, Lauzon, Bishop, Bechard, & Laviolette, 2010). Subsequent electrophysiological experiments showed a dual role for CB1 receptor

transmission within the BLA, with neurons of the mPFC showing an increase in neural firing frequency and bursting during BLA CB1 activation and a decrease in activity during BLA CB1 blockade (Tan et al., 2011). Thus it appears that cannabinoid transmission within the BLA is able to modulate the processing of an emotional fear association via neuronal inputs to areas of the mPFC.

1.2c Interactions with the dopamine system

There are several functional and anatomical interactions between the cannabinoid and DA systems in the CNS. CB1 receptors can be found in brain regions innervated by DA inputs as well as regions that contain high levels of DA receptors (McDonald & Mascagni, 2001; Moldrich & Wenger, 2000). In fact, in the prefrontal cortex CB1 and the D2-receptor subtype are co-localized at terminals of presumed GABAergic synapses, with activation of either receptor causing a suppression of GABA release onto layer V pyramidal cells (Chiu, Puente, Grandes, & Castillo, 2010). Indeed, numerous studies support the finding that CB1 modulation can play an important role in DA activity within the brain. Systemic administration of the CB1 agonist delta-9-tetrahydrocannabinol (THC) or WIN-55, 212-2 (WIN55) has been shown to increase the firing rate and bursting activity of DA neurons in the VTA that project to the PFC (Diana, Melis, & Gessa, 1998; French, Dillon, & Wu, 1997). The reverse effect has also been demonstrated, with intravenous (i.v.) administration of THC or WIN55 increasing the firing rate of PFC pyramidal neurons that project to VTA (Pistis, Porcu, Melis, Diana, & Gessa, 2001). A study by Polissidis et al. (2010) revealed that by administering systemic doses of either THC or WIN55, the biosynthesis of DA was actually increased in regions

like the dorsal striatum, NAc, PFC and amygdala. Furthermore, the amount of phosphorylated DARPP-32 (a DA- and cAMP-regulated neuronal phosphoprotein that can act as an index of DA neurotransmission) increased in the same regions, indicating a key role for cannabinoids in the synthesis and activity of midbrain DA transmission. For example, a well-demonstrated mechanism by which endocannabinoids modulate DA activity is through a retrograde messenger system (Alger, 2002). Release by the depolarized postsynaptic cell enables the diffusion of endocannabinoids such as 2-arachidonoyl-glycerol (2-AG) “backward” across the synaptic cleft where they can bind to the presynaptic cell and cause a suppression of neurotransmitter release. This method of endocannabinoid signalling implies a crucial role for the CB1 receptor in shaping specific patterns of neuronal firing, especially for VTA DA cells. By stimulating the PFC, it was discovered that 2-AG can suppress firing frequency and bursting activity in DA cells of the VTA, indicating that DA neurons can release 2-AG on demand to alter their own firing pattern (Melis et al., 2004). It is evident that exogenous and endogenous cannabinoids can act through both post- and pre-synaptic mechanisms to influence DA activity in various regions throughout the brain. The understanding of this interaction between CB and DA systems becomes increasingly important when considering the role both systems play in specific neuropsychiatric disorders such as schizophrenia.

1.3 Implications for schizophrenia

Schizophrenia is a complicated, multi-faceted disorder with various neurological and behavioural symptoms whose etiology is not yet fully understood. A major facet of the disease comes from the inability to accurately process emotionally salient

information. Where unaffected individuals can attribute appropriate emotional significance to incoming sensory information, patients with schizophrenia have been known to assign an inaccurate amount of importance to specific sensory events, whether externally or internally generated. This dysfunction of emotional processing can manifest itself in the form of “positive” or “negative” symptoms of the disorder. Positive symptoms can include sensory hallucinations and psychotic delusions while negative symptoms include things like anhedonia, apathy and social withdrawal (Berman et al., 1997). Much of the focus on treatment has historically revolved around DA receptors, which will be discussed presently.

1.3a The DA hypothesis of schizophrenia

The DA hypothesis of schizophrenia garnered popularity based on the observations of pharmacological DA manipulation and the effect it had on the psychotic symptoms of the disease. Primarily, D2 receptor antagonists were (and still are) found to be the most effective antipsychotic medication to help alleviate the positive symptoms of schizophrenia (Kapur & Mamo, 2003). Furthermore, it was discovered that drugs causing excessive DA release were able to mimic the psychotic symptoms that many schizophrenic patients experience, in non-schizophrenic individuals (Angrist, Sathananthan, Wilk, & Gershon, 1974). Thus, it was thought that schizophrenia could be characterized as a disease of excessive DA transmission. However, various lines of evidence began to emerge that largely discredited this simplified view, such as the fact that pathological alterations in DA transmission in schizophrenic patients could rarely be confirmed due to the confounds of treatment with antipsychotic medication (Lieberman,

Sheitman & Kinon, 1997). Nonetheless, although the etiology of the disease is not based entirely on DA transmission, the importance of DA in emotional processing is evident and given the correlation between DA, emotional processing, and the deficits seen in schizophrenia, it is clear that this neurotransmitter system plays a crucial role in the positive symptoms of the disorder.

1.3b Cannabinoids and paranoid schizophrenia

As stated previously, CB1 receptor activity has been shown to alter the perception of incoming sensory information, including emotionally relevant stimuli. Those same processing deficits are commonly seen in patients with schizophrenia, specifically the paranoid schizophrenic subtype. Characterized by persistent delusions and/or hallucinations of persecution and suspiciousness, the symptoms of paranoid schizophrenia are similar to the effects experienced by non-schizophrenic individuals after excessive cannabis exposure (Green et al., 2003; Wachtel et al., 2002). Furthermore, there are several pieces of evidence linking the CB1 system to schizophrenia. A long-term study by Andreasson, Engstrom, Allebeck and Rydberg (1987) looked at Swedish conscripts over a 15 year period and found that the risk of developing schizophrenia was positively correlated with the amount of cannabis consumed. They concluded that their data was sufficient to indicate cannabis as an independent risk factor for schizophrenia. Additionally, several studies have reported abnormal levels of the endocannabinoid anandamide in the cerebral spinal fluid of schizophrenic patients (Giuffrida et al., 2004; Leweke, Giuffrida, Wurster, Emrich, & Piomelli, 1999). Abnormal receptor density has been discovered in schizophrenic patients as well. The dorsolateral prefrontal cortex

shows an increase in CB1 receptor binding in non-paranoid schizophrenic patients compared to healthy controls, with paranoid schizophrenics showing an increase in binding over both groups (Dalton, Long, Weickert, & Zavitsanou, 2011; Dean, Sundram, Bradbury, Scarr, & Copolov, 2001). Although not entirely understood, the link between schizophrenia and CB1 dysfunction is distinct and could play an important role in advancing our knowledge of the disease.

1.4 Research Purpose and Hypothesis

Given the interplay between CB1, DA, schizophrenia, and emotional perception, our overarching goal is to elucidate the neural mechanisms by which functional interactions between these systems may lead to aberrant sensory and emotional processing deficits. Understanding these mechanisms will undoubtedly be extremely beneficial for improving our understanding of highly complex, neuropsychiatric disorders. Using a well-established rodent model of associative fear memory, olfactory fear conditioning, we have previously shown that activation of CB1 receptors in mPFC causes a potentiated fear response to a normally non-salient sensory event, both at the single-neuron level and in behaving rats (Laviolette & Grace, 2006a). Although we have identified the effects of mPFC CB1 receptor activation at the cortical level, the purpose of my research project was to examine how CB1 transmission within the mPFC may modulate sub-cortical DA transmission both at the behavioural and neuronal levels of analysis. *The overarching hypothesis of my thesis is that CB1 receptor transmission within the mPFC modulates and controls emotional processing and memory formation*

through functional interactions with sub-cortical DA signaling, via the mesocorticolimbic pathway. I addressed this hypothesis with the following primary AIMS:

1. Using *in vivo* neuronal electrophysiology, examine and characterize the effects of mPFC CB1 transmission on the spontaneous activity patterns of single DA neurons in the VTA

2. Determine how DA transmission is functionally involved in cannabinoid modulation of emotional processing and memory formation at the behavioural systems level.

3. Examine and characterize how functional interactions between the mPFC and VTA mediate the effects of DA and CB1 receptor signaling during emotional processing and memory formation.

2. Materials and Methods

This research project included 14 behavioural groups with N=5-8 rats per group and 102 electrophysiology recordings, with 1-2 cells recorded per rat. The following section describes the materials and methods used during the course of the project.

2.1 Surgical procedure

Male Sprague Dawley rats (300-350g) were used for all surgical procedures. They were anesthetized using an intraperitoneal (i.p.) injection of a ketamine (80mg/ml) xylazine (6mg/ml) mixture and placed in a stereotaxic device. Stainless steel guide cannula (22 gauge) were implanted bilaterally into the VTA or the prelimbic subdivision of the mPFC using the following stereotaxic coordinates, listed in mm. For the VTA (10° angle): from bregma, AP -5.0, L \pm 2.6; from the dural surface, V -8.0. For the mPFC (15° angle): from bregma, AP +2.9, L \pm 1.9; from the dural surface, V -3.0. Cannulae were fixed in place using jeweler's screws and dental acrylic and all rats were given one week of recovery time prior to the beginning of fear conditioning.

2.2 Drug administration

The following drugs were all used during behavioural or electrophysiological experiments: a selective CB1 agonist (WIN 55, 212-2) and antagonist (AM-251), which were dissolved in a 10% DMSO/physiological saline solution, a broad-spectrum DA antagonist (alpha-flupenthixol), a GABA-A antagonist (bicuculline), and a GABA-B antagonist (2-hydroxy-saclofen), which were dissolved in physiological saline (pH adjusted to 7.4). During fear conditioning intra-VTA and intra-mPFC bilateral microinfusions were performed immediately prior to the conditioning phase, with 0.5 μ l total volume per side being delivered via a 28-gauge microinfusion injector over a period of one minute. Microinjectors were left in place for an additional 60 seconds following drug infusion to ensure adequate diffusion from the tip. For groups receiving a pre-treatment of alpha-flupenthixol (α -flu), 0.8mg/kg was delivered via an i.p. injection 2.5

hours prior to the conditioning phase. During electrophysiology recordings, 0.5 μ l of drug was infused over a one minute period via a 26-gauge microinjector implanted into the region of interest.

2.3 Fear conditioning paradigm

We used an olfactory fear conditioning paradigm to test whether rats could recall a learned fear association to an olfactory stimulus that had previously been paired with a footshock. By using subthreshold and suprathreshold footshock levels (0.4 and 0.8mA, respectively) we were able to test whether specific drug treatments could block or potentiate the acquisition of a normal fear memory. In general, a subthreshold footshock fails to produce a normal fear response during later testing while a suprathreshold footshock has been shown to produce a robust fear memory (Laviolette & Grace, 2006a). Two distinct environments were used during experiments. Environment A was a 30" x 30" Plexiglass box with black spots on a white background and environment B was a 30" x 30" Plexiglass box with black and white stripes. The designated "shock" environment had a metallic grid shock floor while the designated "test" environment had a smooth grey Plexiglass floor. The environments were assigned as "shock" and "test" in a counterbalanced manner such that all rats with "shock" environment A were tested in environment B and all rats with "shock" environment B were tested in environment A. On day one (habituation phase) rats were given sham microinfusions into either the mPFC or the VTA and habituated to both "shock" and "test" environments located in a sound-attenuated room, spending 30 minutes in each. On day two (conditioning phase)

rats were returned to the room, drug infusions were performed, and the animal was placed in the previously assigned “shock” environment. Two odors were delivered during the course of conditioning, almond and peppermint (Clubhouse brand). One odor was presented with a footshock (CS+) and the other was presented in the absence of a footshock (CS-). After one minute in the “shock” environment, the CS- odor was presented for 20s. Two minutes later, the CS+ odor was presented for 19s followed by a 1s footshock delivered through the metallic grid shock floor. This cycle was repeated five times after which the rats were returned to their home cages. On day three (testing phase) rats were placed in the previously assigned “test” environment. They were given one minute to explore the environment before the odor presentation began, during which time baseline levels of freezing and exploratory behaviour were recorded. Both CS+ and CS- odors were then presented for 5 minutes each and the amount of time the rat spent freezing was recorded. Freezing is a well-known behavioural measure of fearfulness and was defined as the absence of all movement aside from respiration. We also examined exploratory scores in response to CS+ and CS- presentations, as described previously (Lauzon et al., 2009; Steven R Laviolette et al., 2005). We assigned a score based on the following scale, with each minute of the 5 minute odor presentation being scored individually/separately: 0, no locomotion; 1, ambulation across one side of the testing chamber; 2, ambulation across two sides; 3, exploration of the full perimeter of the testing chamber; and 4, exploration of the center and entire perimeter of the test chamber. Thus, each rat received a score for both exploratory behaviour and percentage of time spent freezing during the test phase to CS+ and CS- presentations.

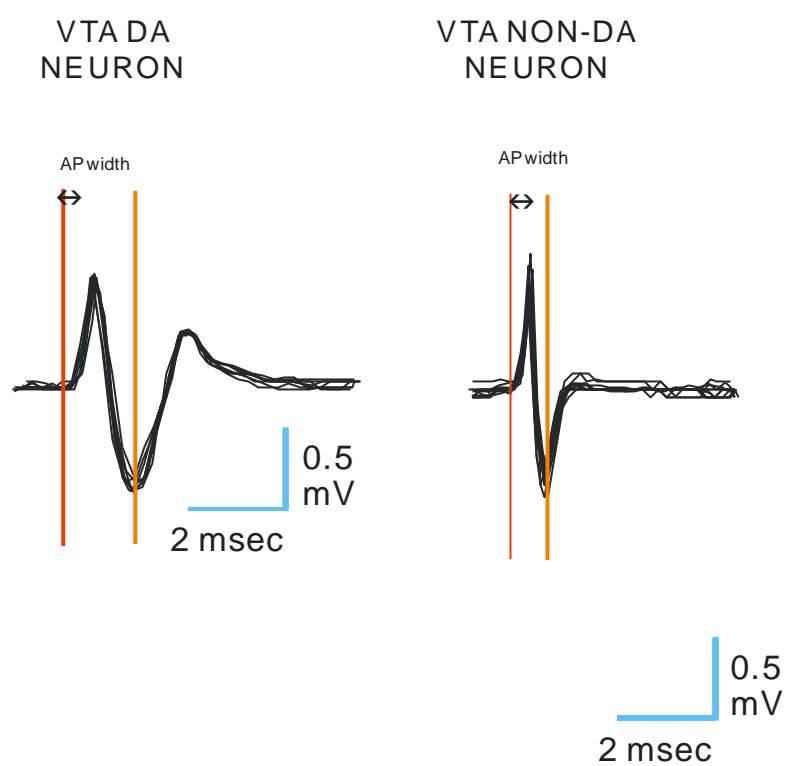
2.4 Electrophysiology procedure

We performed single-unit extracellular electrophysiology recordings using conventional methods as described in Laviolette et al. (2005). Our two areas of interest were the PLC of the mPFC and the VTA, with cell recordings in one region corresponding to drug infusion into the other. We distinguished mPFC pyramidal cells based on firing frequency, with pyramidal neurons exhibiting a spontaneous firing rate of less than 4Hz. For VTA recordings we isolated DA neurons using criteria as described previously by Grace and Onn (1989). DA neurons were identified according to well established electrophysiological features: (1) a relatively longer action potential width (>2.5 ms) calculated automatically by existing software, a triphasic (+ /- / +) waveform consisting of a notch on the rising phase followed by a delayed afterpotential; (2) a slow, irregular or bursting firing pattern, and (3) a spontaneous firing rate of 2–5 Hz or less. In contrast, non-DAergic, VTA neurons were characterized based upon previously reported criteria: (1) a narrow action potential width (< 1 msec), (2) a biphasic waveform (- / +) and (3) relatively fast firing rates (typically ~ 10 -20 Hz) and the absence of bursting activity (See Figure 1). For all recording experiments male Sprague Dawley rats (300–350 g) were anesthetized using urethane (Sigma; 1.4 –1.5 mg/kg, i.p.) and placed in a stereotaxic device. Body temperature was monitored with a rectal temperature probe and maintained at 37°C. Incisions were made in the scalp to expose the skull, burr holes were drilled, and the dura overlying the VTA and mPFC was removed. A 26-gauge microinjector (Hamilton, 10ul) was loaded with the appropriate drug treatment and slowly lowered into the region of interest (either VTA or mPFC) using the following coordinates, listed in mm. For the VTA (no angle): from bregma, AP -5.5, L ± 0.5 ; from

the dural surface, V -6.5-8.0. For the mPFC (20° forward angle): from bregma, AP +4.0, L \pm 0.9; from the dural surface, V -2.5-3.0. Borosilicate glass was used to pull electrodes with an impedance, on average, between 6 and 8 M Ω . Electrodes were then filled with 2% Pontamine Sky Blue and slowly lowered into the VTA or mPFC using the coordinates described above. The extracellular signals were amplified using a MultiClamp 700B amplifier (Molecular Devices) and recorded through a Digidata 1440A acquisition system (Molecular Devices) using pClamp 10 software. Recordings were filtered at 1kHz and sampled at 5kHz. Once a cell had been located and proven stable we recorded 6-10 minutes of baseline activity before infusing drugs into the region of interest. A total volume of 0.5ul of the drug was infused over a period of one minute after which time the recording continued for at least 20 minutes, unless the cell became unstable during that time frame. For analysis, we compared the frequency of mPFC or VTA neuronal cell firing frequency for the 5 minute period before and 5 minute period after drug infusion had occurred.

Figure Caption 1

Typical waveforms of both DA and non-DA neurons found within the VTA. Note the differences in height, width and after-potential.

**Figure 1**

2.5 Histology

Following completion of experiments all rats were overdosed on Euthanyl (Sodium Pentobarbital, 240mg/kg, i.p.) and perfused using an isotonic saline solution followed by 10% formalin. All brains were extracted and placed in the formalin solution for at least 24 hours before being placed in a formalin-sucrose solution for at least one week before slicing. Brains were sliced using a cryostat at 40µm and mounted onto slides. They were stained with cresyl violet and the placements for mPFC and VTA were verified using light microscopy. Any rats showing cannula placements outside the boundaries defined by Paxinos and Watson (2005) were excluded from data analysis.

2.6 Statistical analyses

Behavioral and/or electrophysiological data were analyzed with one- or two-way ANOVA or Student's t tests where appropriate. Post hoc analyses were performed with Fisher's LSD tests or Newman–Keuls tests.

3. Results

3.1 The effect of intra-mPFC activation of CB1 receptors on the firing frequency of dopaminergic neurons in the VTA

To study the effect of cortical cannabinoid transmission on subcortical DA activity, *in vivo* extracellular electrophysiology was performed in the VTA during CB1 receptor activation in the mPFC. Two doses of the CB1 agonist, WIN 55 (low: 50ng/0.5µl; high: 500ng/0.5µl), were used during experiments, as well as a low dose of

the CB1 antagonist, AM-251 (50ng/0.5 μ l). Once a VTA DA cell was isolated a baseline firing frequency was established, after which point the intra-mPFC drug administration occurred, followed by a further 30 minutes of recording. We sampled a total of $n = 65$ VTA neurons during intra-mPFC infusion of low-dose WIN55 ($n=38$), high-dose WIN55 ($n=19$) and a small control group using AM-251 ($n=8$). Results from all sampled neurons across the three drug treatments are summarized in Figure 2. For rats receiving the low dose of intra-mPFC WIN55, 50% of neurons showed increased activity, 32% showed decreased activity, and 18% demonstrated no change in activity levels. Interestingly, the opposite effect was seen for rats receiving the high dose of intra-mPFC WIN55, where 26% of neurons showed increased activity, 63% showed decreased activity, and 11% demonstrated no change in activity levels. Thus, across both doses of intra-mPFC WIN55 we observed a biphasic effect where a plurality of cells showed an increase in spontaneous neuronal activity patterns after infusion of a low-dose CB1 agonist and a plurality of cells showed a decrease in spontaneous neuronal activity patterns after infusion of a high-dose CB1 agonist. In Figure 3 we present representative rastergrams showing firing frequency patterns during VTA recordings. Figure 3A shows a typical increase pattern after mPFC infusion of low-dose WIN55 while Figure 3B shows a typical decrease pattern after mPFC infusion of high-dose WIN55.

Figure Caption 2

Summary of the effects of intra-mPFC WIN55 (50 & 500ng/0.5µl) on spontaneous firing frequency on sampled neurons recorded in the VTA. A small control group also received intra-mPFC WIN55 (50ng/0.5µl) + AM-251 (50ng/0.5µl).

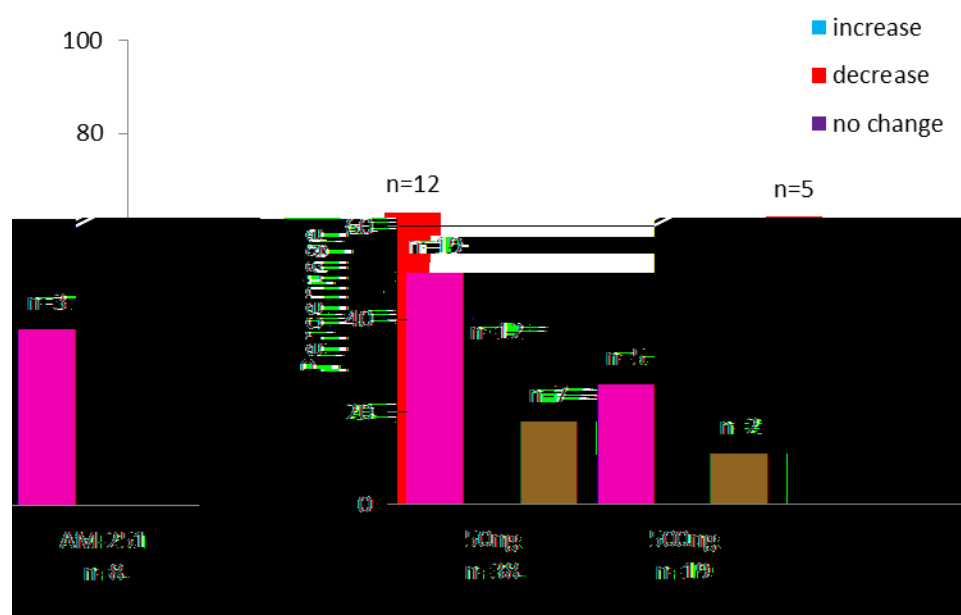


Figure 2

Figure Caption 3

Representative rastergrams showing firing frequency patterns of sampled VTA neurons during mPFC drug infusion. For these and subsequent rastergrams bin width is equal to 1000ms.

(A) Representative VTA neuronal recording sample demonstrating a typical increase in neuronal activity after intra-mPFC WIN55 (50ng/0.5µl).

(B) Representative VTA neuronal recording sample demonstrating a typical decrease in neuronal activity after intra-mPFC WIN55 (500ng/0.5µl).

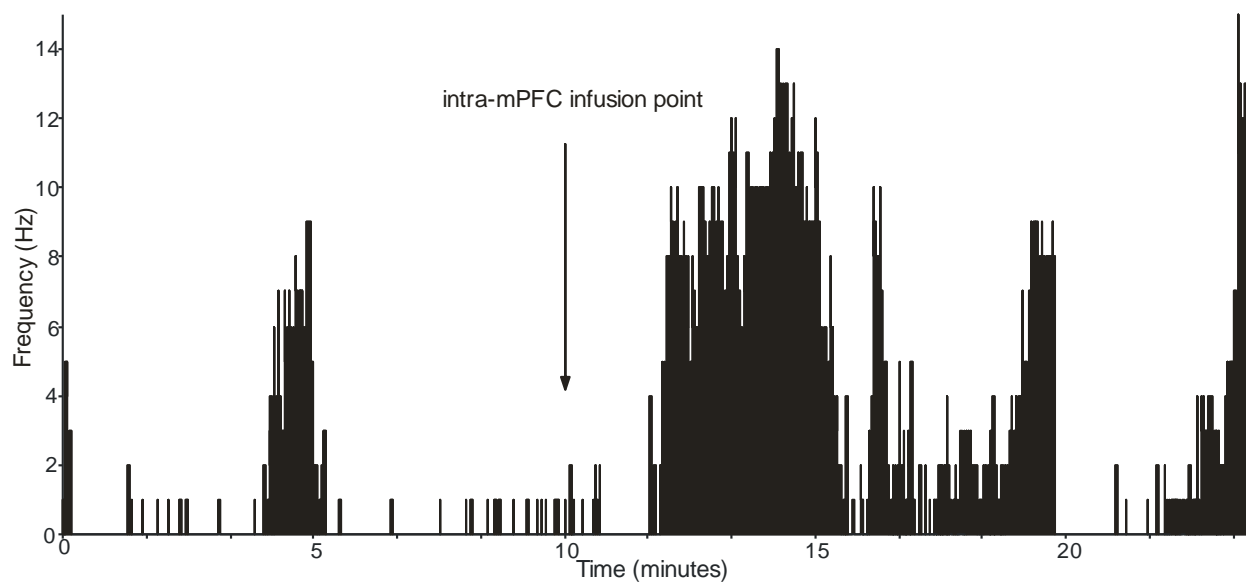
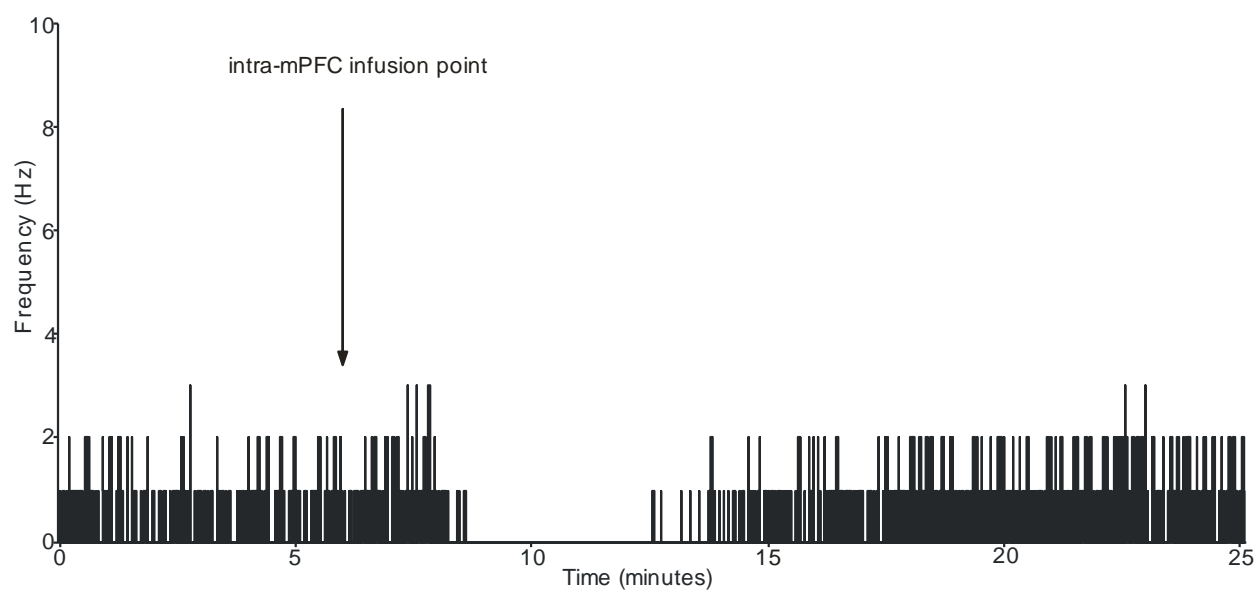
A**B****Figure 3**

Figure 4A displays group data for *in vivo* recordings performed in the VTA during intra-mPFC WIN55 administration for the subpopulations of VTA neurons that demonstrated an increase in firing frequency after drug infusion. Two-way ANOVA revealed a significant main effect of time ($F_{(1,75)} = 30.21, p < 0.01$). Post hoc analysis revealed that VTA neuronal firing frequency was significantly increased relative to baseline after infusion of the low-dose CB1 agonist ($p < 0.01$), but not after infusion of the high-dose CB1 agonist ($p > 0.05$). Thus, it appears that the low dose of WIN55 was able to significantly increase firing frequency in a plurality of VTA cells after intra-mPFC drug infusion. While the high dose of WIN55 did increase firing frequency in a small subset of VTA cells the effect was non-significant.

Subpopulations of VTA cells also showed a decrease in firing frequency after mPFC drug infusion of WIN55 (Figure 4B). Two-way ANOVA revealed a significant main effect of time ($F_{(1,47)} = 122.23, p < 0.01$). Post hoc analysis revealed that VTA neuronal firing frequency was significantly decreased relative to baseline after infusion of both doses of CB1 agonist (p 's < 0.01). Thus, the high dose of WIN55 was able to significantly decrease firing frequency in a large majority of VTA cells after intra-mPFC drug infusion while the low dose of WIN55 was also able to significantly decrease firing frequency in a smaller subset of VTA cells. A representative microphotograph of a mPFC injection site is presented in Figure 5A, while a VTA electrode recording site is presented in Figure 5B. A schematic representation of VTA recording sites is shown in Figure 5C for cells showing an increase in firing frequency at the low dose and a decrease in firing frequency at the high dose.

Figure Caption 4

Effects of intra-mPFC WIN55 on spontaneous neural activity in the VTA. For this figure, and subsequent figures, * indicates $p < 0.05$ and ** indicates $p < 0.01$

(A) Intra-mPFC infusions of WIN55 cause a significant increase in firing frequency of recorded VTA DA neurons at the 50ng dose, but not the 500ng dose.

(B) Both 50ng and 500ng doses of intra-mPFC WIN55 cause a significant decrease in firing frequency for recorded VTA DA neurons.

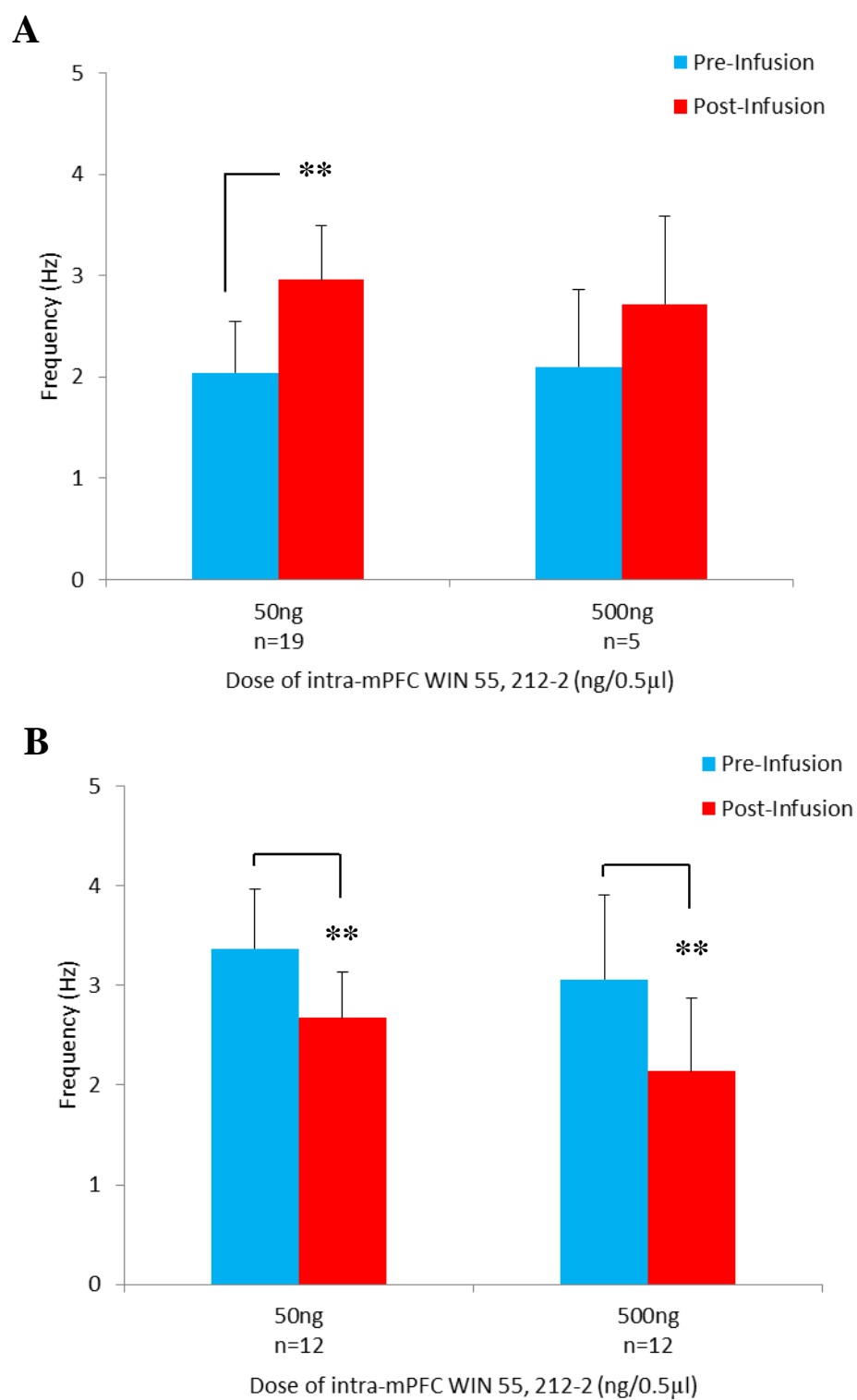
**Figure 4**

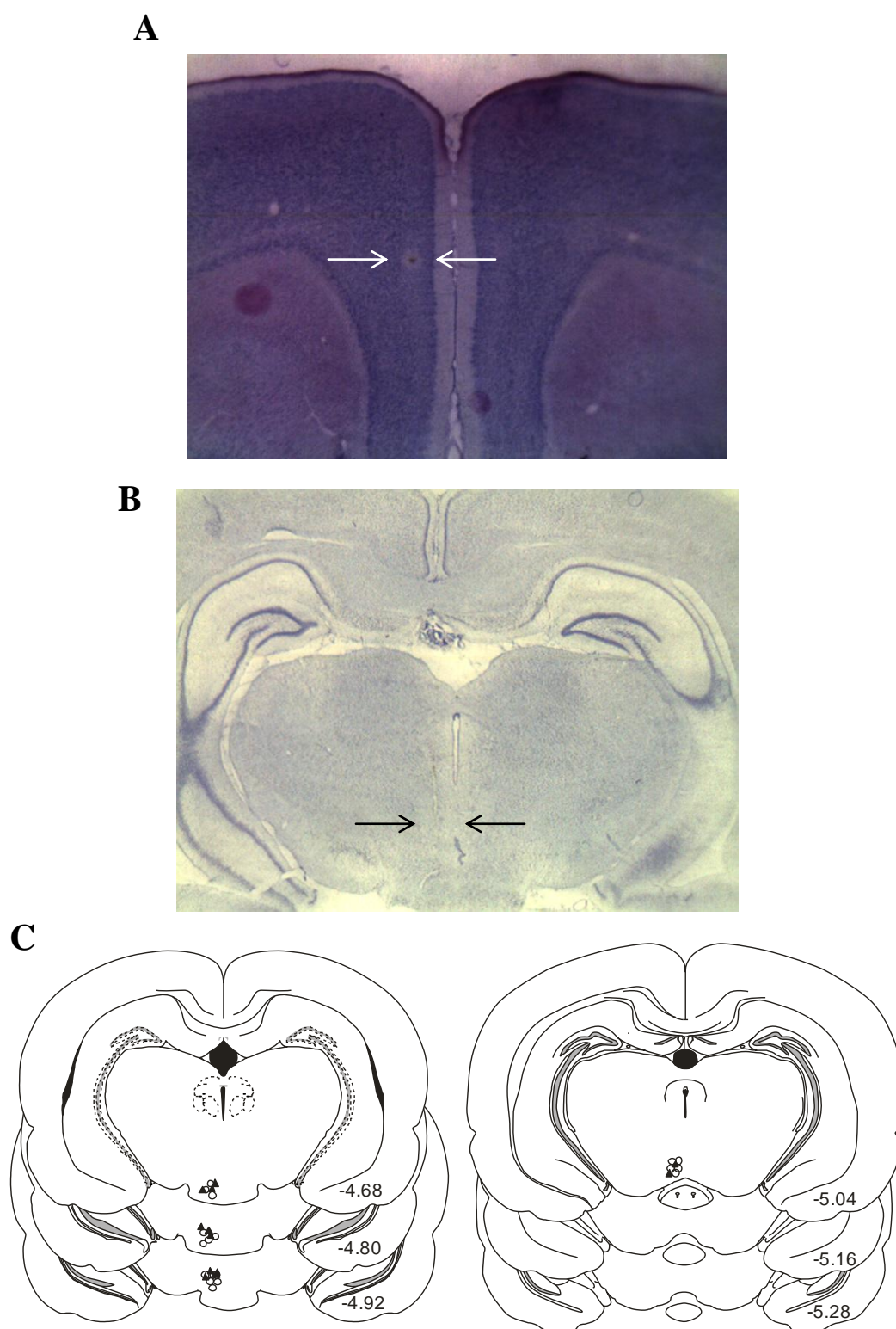
Figure Caption 5

Histology of VTA recording sites.

(A) Microphotograph of an intra-mPFC injection site. Arrows indicate microinjector tip.

(B) Microphotograph of an intra-VTA electrode recording site. Arrows indicate electrode tip.

(C) A schematic representation showing VTA recording sites for cells that showed an increase in firing frequency at the 50ng WIN55 dose (▲) and cells that showed a decrease in firing frequency at the 500ng WIN55 dose (○).

**Figure 5**

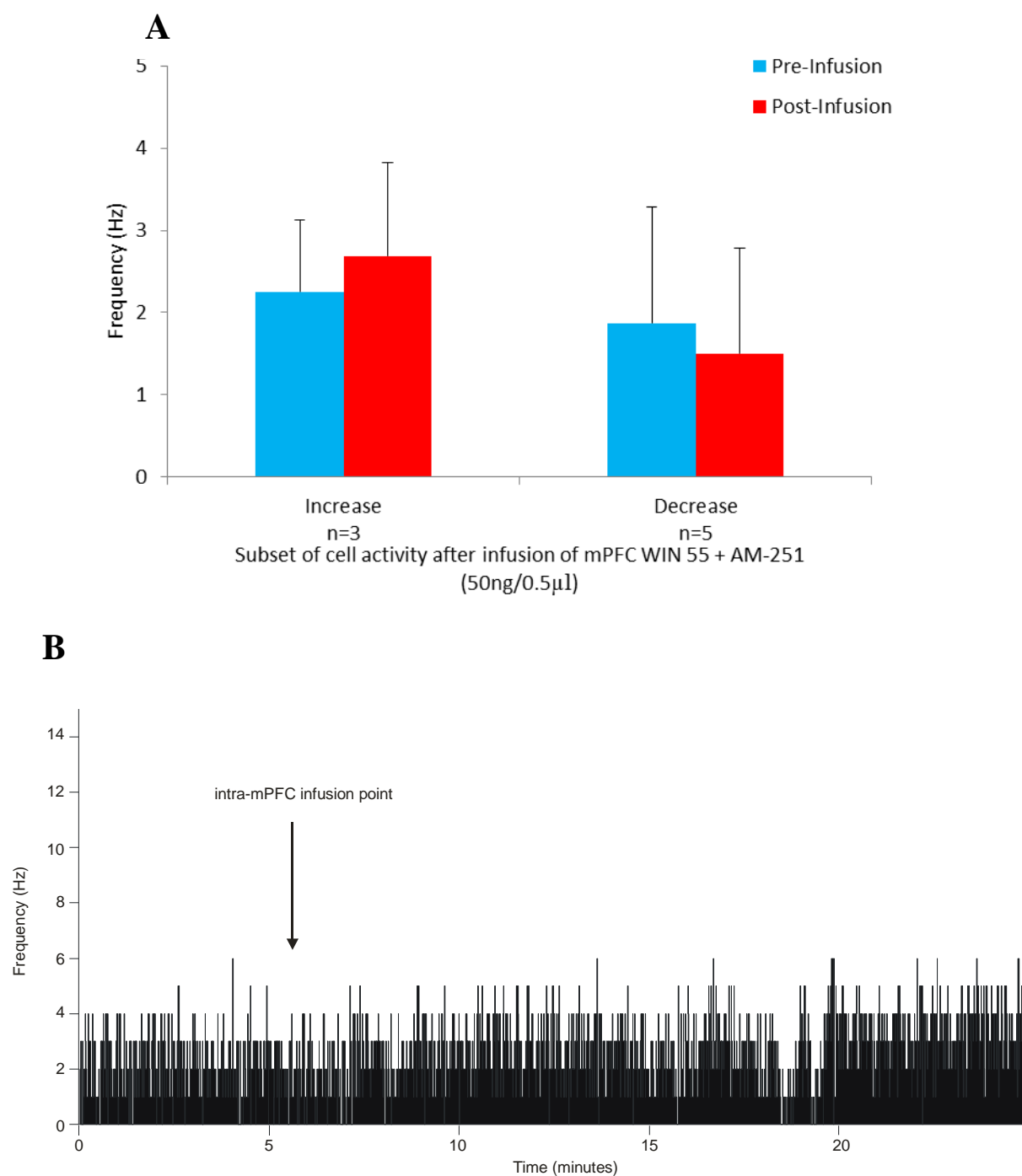
To demonstrate pharmacological specificity for the effects of intra-mPFC WIN55, a separate control experiment was performed using the lower dose of intra-mPFC WIN55 mixed with AM-251 (50ng/0.5µl), a highly selective CB1 receptor antagonist. In this study, 38% of isolated DA neurons showed a trend towards increased activity while 62% showed a trend towards decreased activity. Two-way ANOVA revealed no significant differences in firing rate after infusion for either subpopulation of cells ($F_{(1,15)} = 0.001$, $p > 0.05$) (Figure 6A). Thus, intra-mPFC co-infusion of WIN55 + AM-251 was able to block the increase in firing frequency observed in a plurality of cells after intra-mPFC infusion of the low-dose WIN55 alone. A representative rastergram is shown in Figure 6B, demonstrating a typical firing pattern after mPFC infusion of low-dose WIN55 + AM-251. Thus, the effects of intra-mPFC WIN55 are mediated through a CB1-dependent substrate, as the co-administration of a selective CB1 receptor antagonist was able to block the excitatory effects on sub-cortical DAergic neuron activity in the majority of sampled neurons.

Figure Caption 6

The results of AM-251 control experiments.

(A) Intra-mPFC infusions of WIN55 (50ng/0.5µl) + AM-251 (50ng/0.5µl) did not cause a significant increase or decrease in firing frequency for sampled VTA DA neurons.

(B) Representative VTA neuronal recording sample demonstrating a typical firing pattern after intra-mPFC WIN55 + AM-251.

**Figure 6**

3.2 Activation of CB1 receptors in VTA excites intra-mPFC pyramidal neurons.

As noted previously, the mPFC and VTA show functional interactions in a bi-directional manner, via mPFC>VTA descending or VTA>mPFC ascending projections (Sesack & Carr, 2002). In an effort to further characterize the role of CB1 activity within the VTA-mPFC circuit we performed the reciprocal experiments as explained above, with *in vivo* electrophysiology being performed in the mPFC during CB1 receptor activation in the VTA. As before, two doses of the CB1 agonist, WIN55 (low: 50ng/0.5µl; high: 500ng/0.5µl) were used during experiments. We sampled a total of $n = 37$ mPFC neurons during intra-VTA infusion of low-dose ($n=19$) and high-dose ($n=18$) WIN55. Results from all sampled neurons across both drug treatments are summarized in Figure 7. For rats receiving the low dose of intra-VTA WIN55, 58% of neurons showed increased activity and 42% showed decreased activity. A similar effect was seen for rats receiving the high dose of intra-VTA WIN55, where 50% of neurons showed increased activity, 28% showed decreased activity, and 22% demonstrated no change in activity levels. Thus, across both doses of intra-VTA WIN55 administration CB1 activation was able to increase spontaneous neural activity in a plurality of sampled neurons within the mPFC.

Figure 8A displays group data for *in vivo* recordings performed in the mPFC during intra-VTA WIN55 administration for the subpopulations of mPFC neurons that demonstrated an increase in firing frequency after drug infusion. Two-way ANOVA revealed a significant main effect of time ($F_{(1,43)} = 43.34, p < 0.01$). Post hoc analysis revealed that mPFC neuronal firing frequency was significantly increased relative to baseline after infusion of both the low-dose and high-dose CB1 agonist (p 's < 0.01). It

appears that both doses of WIN55 were able to significantly increase spontaneous neural activity in a majority of sampled cells within the mPFC.

Subpopulations of mPFC cells also showed a decrease in firing frequency after intra-VTA drug infusion of WIN55 (Figure 8B). Two-way ANOVA revealed a significant main effect of time ($F_{(1,31)} = 20.62, p < 0.01$). Post hoc analysis revealed that mPFC neuronal firing frequency was significantly decreased relative to baseline after intra-VTA infusion of both doses of CB1 agonist (p 's < 0.05). Thus, both low and high doses of WIN55 were able to significantly decrease firing frequency in a smaller subpopulation of sampled cells in mPFC.

Figure Caption 7

(A) A schematic representation showing PFC recording sites during intra-VTA infusion of 500ng WIN55.

(B) Summary of the effects of intra-VTA WIN55 (50 & 500ng/0.5µl) on spontaneous firing frequency for sampled neurons recorded in the mPFC.

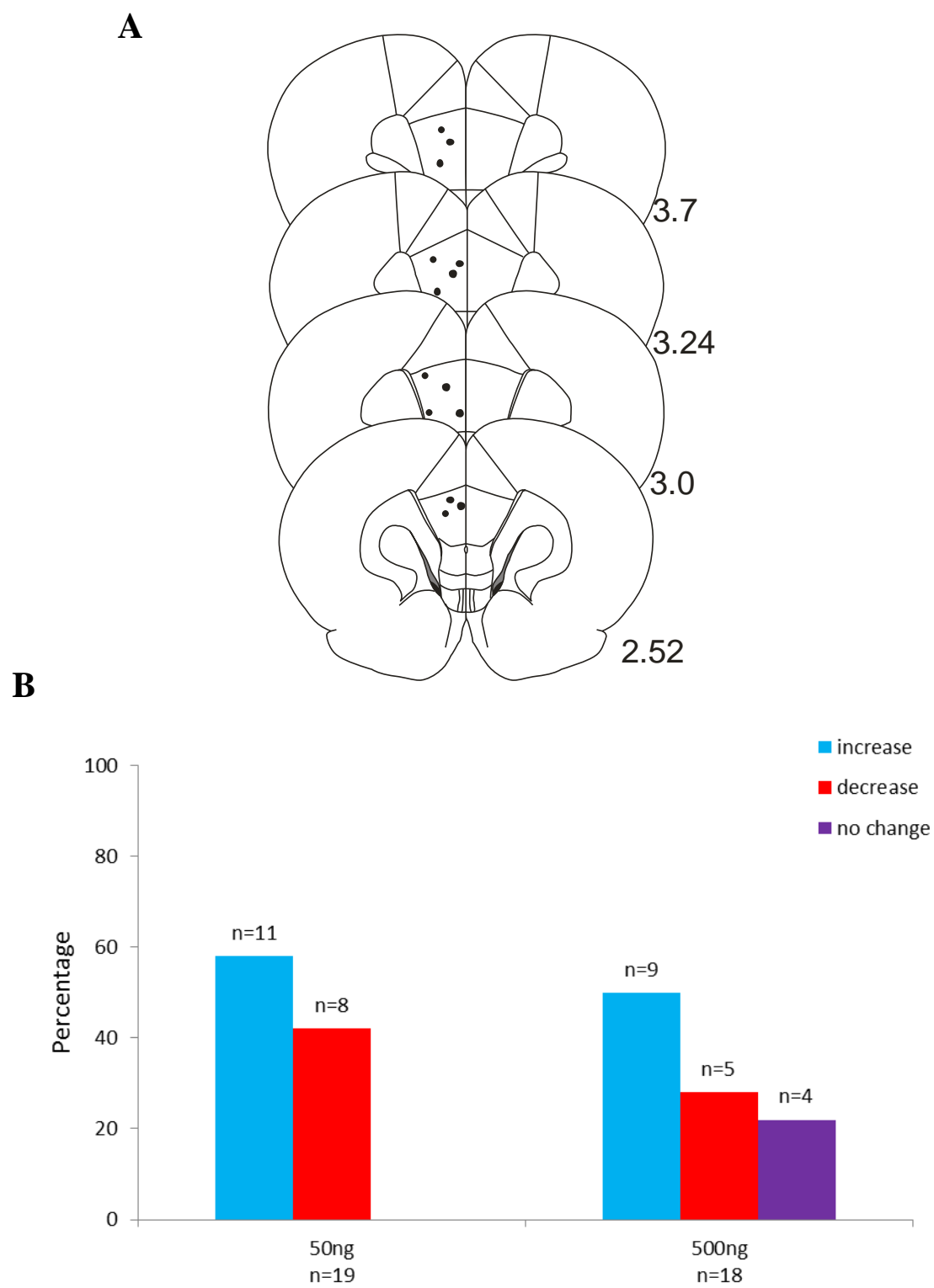
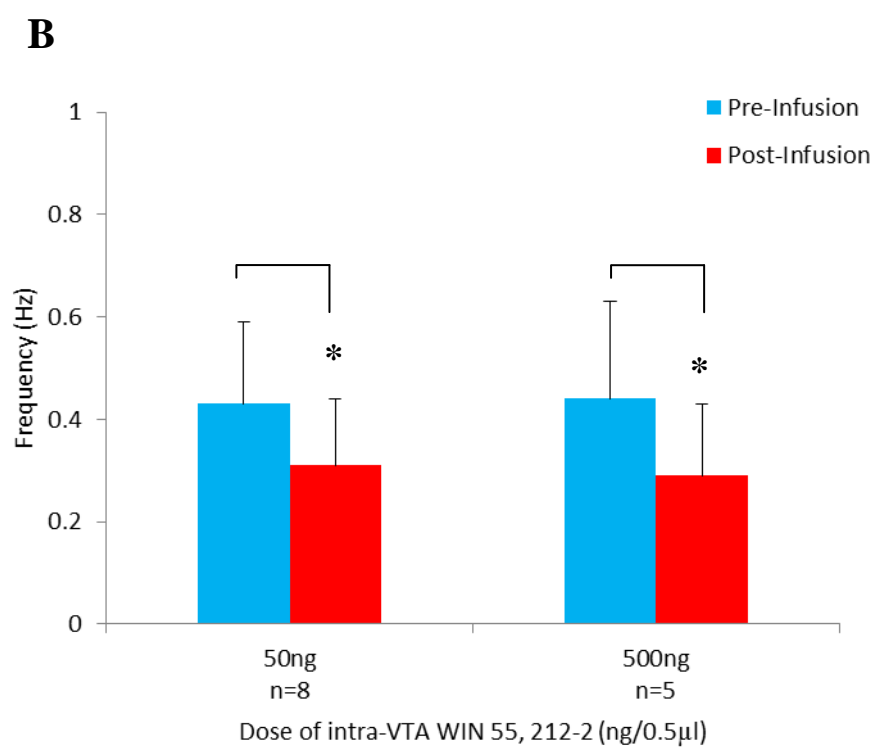
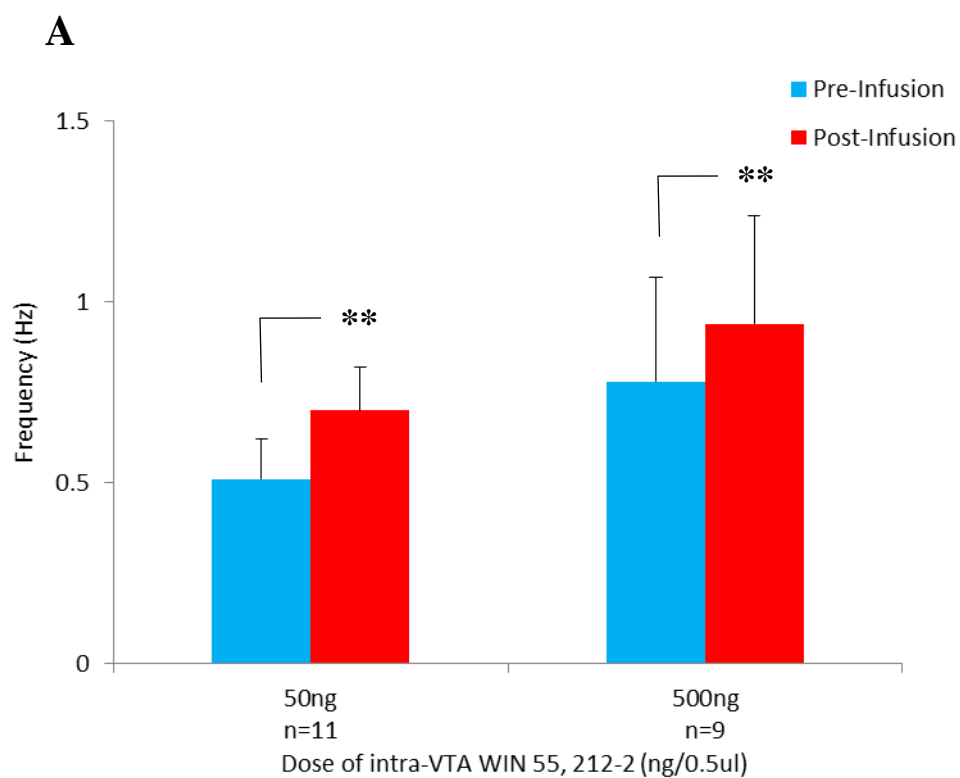
**Figure 7**

Figure Caption 8

Effects of intra-VTA WIN55 on spontaneous neural activity in the mPFC.

(A) Intra-VTA infusions of WIN55 cause a significant increase in firing frequency of recorded mPFC neurons at both the 50ng and 500ng dose.

(B) Both 50ng and 500ng doses of intra-VTA WIN55 cause a significant decrease in firing frequency for recorded mPFC neurons.

**Figure 8**

3.3 Potentiation of fear acquisition by a low dose CB1 agonist is blocked by a dopamine receptor antagonist

Using our olfactory fear conditioning paradigm, the effects of cortical cannabinoid transmission on fear memory acquisition were tested. Previously, it has been shown that the activation of CB1 receptors in the mPFC by a low dose of WIN 55 (50ng/0.5µl) can potentiate a fear response to a normally subthreshold level of footshock (Laviolette & Grace, 2006a). In an attempt to further investigate the underlying mechanisms of this phenomenon, a low dose of WIN 55 was infused into the mPFC during subthreshold fear conditioning. We then challenged the role of DA transmission in this potentiated fear memory response by using a broad-spectrum DA antagonist both locally (α -flu, 0.1µg or 1µg/0.5µl) and systemically (α -flu, 0.8mg/kg). For local application, a low dose of WIN55 was mixed with either a low or high dose of α -flu (0.1µg or 1µg/0.5µl) and infused into the mPFC immediately prior to the conditioning phase. For systemic application, α -flu (0.8mg/kg) was administered via an i.p. injection approximately 2.5 hours prior to the conditioning phase. Infusion of WIN55 (50ng/0.5µl) into the mPFC took place immediately prior to the conditioning phase as usual.

The results of the olfactory fear conditioning experiments revealed that bilateral microinfusions of WIN55 into the mPFC were able to potentiate normal freezing behaviour to a subthreshold level of footshock. This fear memory potentiation was subsequently blocked by intra-mPFC co-infusion of two doses of α -flu (0.1µg/0.5µl & 1µg/0.5µl) (Figure 9A).

For groups receiving intra-mPFC WIN55/ α -flu co-administration, analysis of conditioned freezing behaviour with two-way ANOVA revealed a significant interaction

between group and treatment ($F_{(3,49)} = 8.37, p < 0.01$) on the amount of time spent freezing to CS+ vs. CS- presentations. Post hoc analysis revealed that rats receiving intra-mPFC WIN55 demonstrated significant levels of conditioned fear, spending a greater amount of time freezing to the CS+ presentation than the CS- ($n=6, p < 0.01$). In contrast, the groups receiving intra-mPFC saline ($n=7$), or WIN55 mixed with the lower ($0.1\mu\text{g}, n=6$) or higher ($1\mu\text{g}, n=6$) doses of α -flu showed no behavioural memory of fear acquisition, with equal amounts of time spent freezing to the CS+ and CS- presentations (p 's > 0.05).

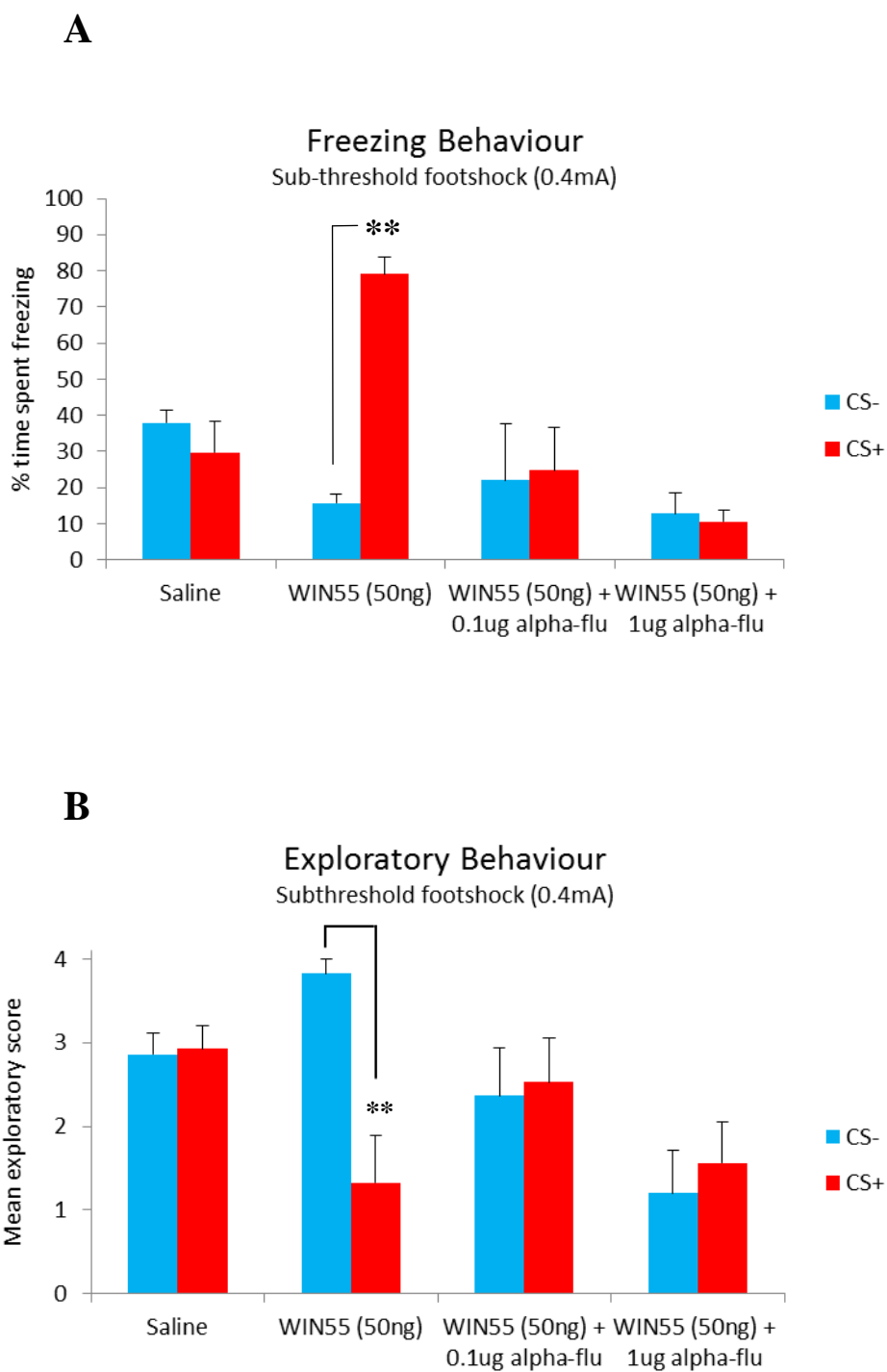
Similarly, two-way ANOVA conducted on spontaneous exploratory behaviour during presentations of CS+ and CS- revealed a significant interaction between group and treatment ($F_{(3, 49)} = 4.85, p < 0.01$). Post hoc analysis showed rats treated with intra-mPFC WIN55 displayed significantly lower levels of exploratory behaviour during the CS+ presentation relative to the CS- ($p < 0.01$) (Figure 9B). In contrast, there was no significant difference in exploratory behaviour during CS+ and CS- presentations for groups receiving intra-mPFC saline or WIN55 mixed with either dose of α -flu (p 's > 0.05).

Figure Caption 9

Effects of intra-mPFC WIN55 on the expression of subthreshold olfactory fear memory.

(A) A subthreshold level of footshock (0.4mA) produces no fear response in saline treated control animals. However, bilateral intra-mPFC infusions of WIN55 (50ng/0.5µl) potentiate normal fear memory acquisition, with rats spending an increased amount of time freezing to CS+ presentations. This effect was blocked with local administration of both doses (0.1 + 1.0µg/0.5µl) of the DA antagonist, α -flu.

(B) Similarly, intra-mPFC WIN55 was able to attenuate normal exploratory behaviour to CS+ presentations. This effect was blocked with local administration of α -flu.

**Figure 9**

To further investigate the effect of DA inhibition on the potentiation of emotional associative learning during intra-mPFC cannabinoid activation, we included an experiment wherein rats received a systemic dose of α -flu (0.8mg/kg; i.p.). Similar to our results with intra-mPFC administration of α -flu, the results of this olfactory fear conditioning experiment revealed that systemic administration of α -flu was able to block the potentiated fear response seen during intra-mPFC CB1 activation (Figure 10A).

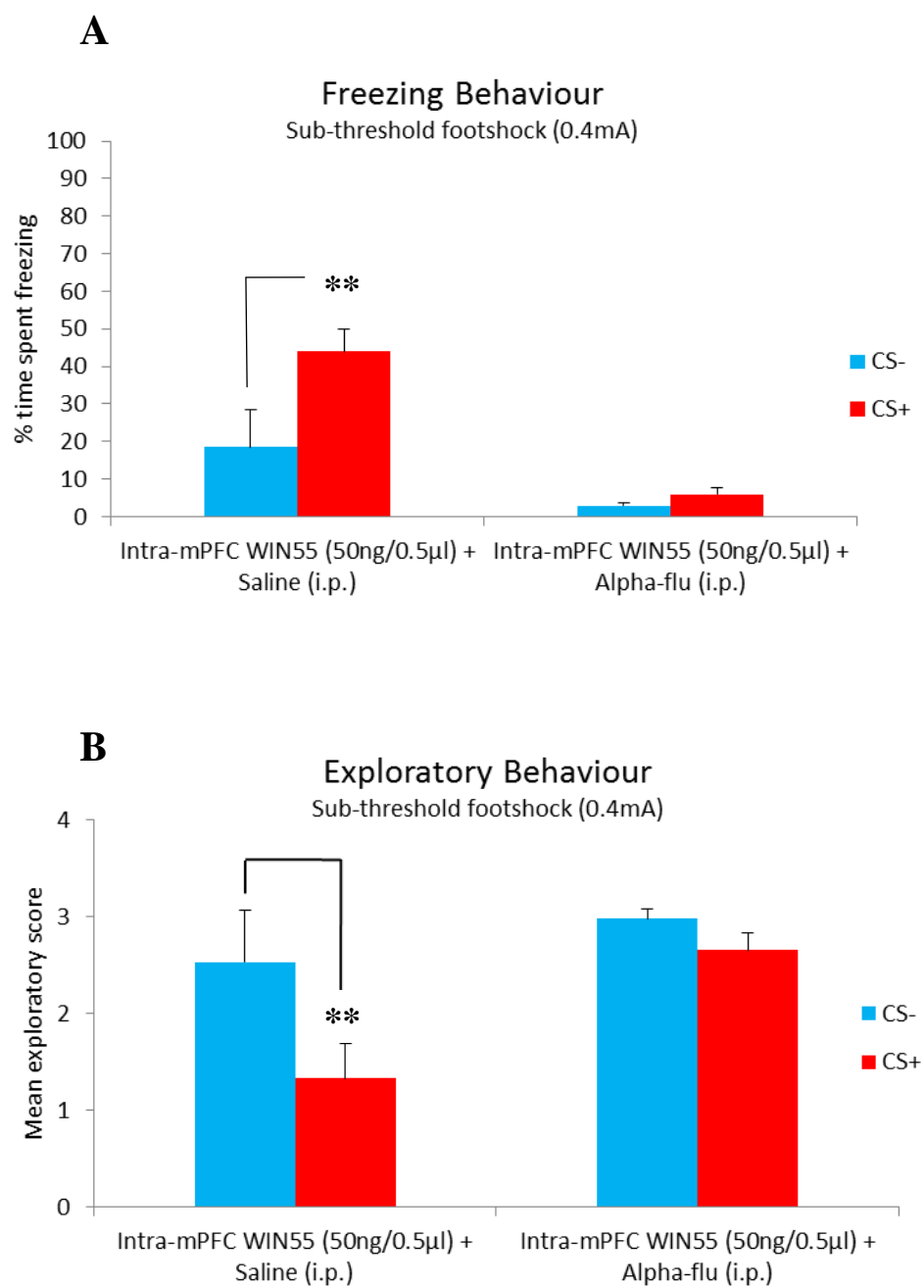
Analysis of conditioned freezing behaviour with two-way ANOVA revealed a significant interaction between group and treatment ($F_{(1,27)} = 4.93, p < 0.05$) on the amount of time spent freezing to CS+ vs CS- presentations. Post hoc analysis revealed that rats receiving intra-mPFC WIN55 with systemic saline demonstrated significant levels of conditioned fear, spending a greater amount of time freezing to the CS+ presentation than the CS- ($n=6, p < 0.01$). In contrast, rats receiving intra-mPFC WIN55 with systemic α -flu showed no behavioural memory of fear acquisition, with equal amounts of time spent freezing to the CS+ and CS- presentations ($n=8, p > 0.05$). Two-way ANOVA of exploratory behaviour scores during CS+ and CS- presentations revealed a significant main effect of treatment ($F_{(1,27)} = 8.71, p < 0.01$). Post hoc analysis showed rats receiving intra-mPFC WIN55 with systemic saline displayed significantly lower levels of exploratory behaviour during the CS+ presentation relative to the CS- ($p < 0.05$) (Figure 10B). Rats receiving intra-mPFC WIN55 with systemic α -flu showed no significant difference in exploratory behaviour during CS+ and CS- presentations ($p > 0.05$).

Figure Caption 10

Effects of intra-mPFC WIN55 with systemic α -flu on the expression of olfactory fear memory.

(A) Rats receiving bilateral intra-mPFC infusions of WIN55 (50ng/0.5 μ l) with saline pre-treatment showed a potentiation of normal fear memory acquisition, spending an increased amount of time freezing to CS+ presentations. This effect was blocked in rats receiving bilateral intra-mPFC infusions of WIN55 with α -flu pre-treatment.

(B) Similarly, intra-mPFC WIN55 was able to attenuate normal exploratory behaviour to CS+ presentations. This effect was blocked with systemic administration of α -flu.

**Figure 10**

To rule out the possibility of altered nociceptive processing mediated by α -flu administration we performed a separate group of control experiments to measure footshock sensitivity in saline vs. α -flu treated rats. Animals received a systemic injection of either saline or α -flu (0.8mg/kg) 2.5 hours prior to the control experiment. Testing took place 2.5 hours later when they were placed in a clear Plexiglass environment with a grid shock floor. We administered a subthreshold level of footshock once every 60 seconds for five minutes and measured sensitivity to footshock over three separate variables: mean exploratory score for each minute following footshock, number of jumps in response to footshock, and amount of defecation (in pieces) during footshock session. All of these behavioural indices of footshock sensitivity are among those reported as reliable indicators of fear to the presentation of a footshock stimulus (Antoniadis & McDonald, 1999). Comparing saline versus α -flu treated rats revealed no significant differences in the number of jumps in response to footshock ($t_{(12)} = 0.00$, $p > 0.05$) (Figure 11A). Similarly, there were no significant differences in the amount of defecation during the footshock session ($t_{(12)} = 0.59$, $p > 0.05$) (Figure 11B). We did observe a significant difference in the exploratory behaviour scores between saline and α -flu treated rats, with saline treated animals having significantly higher exploratory scores ($t_{(6,9)} = 4.27$, $p < 0.01$) (Figure 11C). This observation is to be expected as α -flu is a known cataleptic and would therefore impair motor behaviour as compared to the saline treated group. However, as testing during our previous experiment took place 24 hours after α -flu administration, the rats were all tested in a drug-free state and were thus capable of normal motor movement. As the jumping and defecation scores from our sensitivity control experiment indicate, the α -flu treated rats were still experiencing normal sensitivity patterns associated with

the subthreshold footshock. We can therefore conclude that systemic α -flu administration does not cause significant impairments in nociceptive processing to footshock presentation.

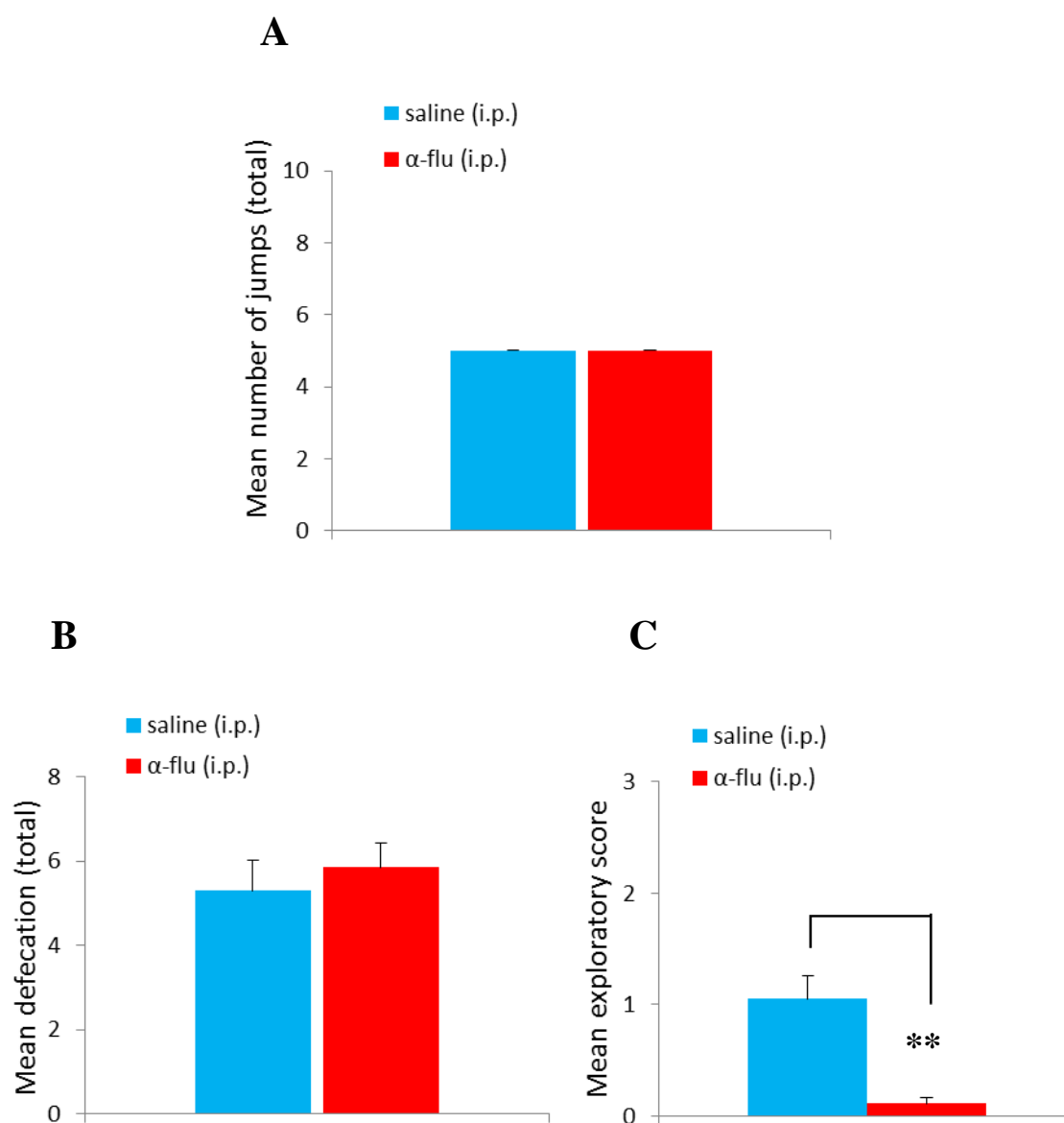
Figure Caption 11

The results of footshock sensitivity testing.

(A) Footshock sensitivity testing revealed no significant differences between control and α -flu groups for the average number of jumps per trial in response to footshock.

(B) There were no significant differences between saline and α -flu groups for the average amount of defecation per trial.

(C) There was a significant difference observed in exploratory behaviour between saline and α -flu groups. This can be attributed to the known cataleptic properties of α -flu and would not be a factor for our main experimental group as testing took place in a drug-free state.

**Figure 11**

3.4 The effect of high dose CB1 activation on fear acquisition

To examine the effect of a higher dose of the CB1 agonist, WIN 55, 212-2 (500ng/0.5ul), on fear memory acquisition, olfactory fear conditioning was performed using a suprathreshold footshock level (0.8 mA) which normally produces a robust freezing response (Lauzon et al., 2009; Laviolette & Grace, 2006). As before, all intra-mPFC infusions were made immediately prior to the behavioural conditioning phase.

In direct contrast to the potentiating effects of the lower dose of intra-mPFC WIN55, the results of the olfactory fear conditioning experiments revealed that bilateral microinfusions of high-dose WIN55 (500 ng/0.5 µl) into the mPFC blocked normal freezing behaviour to a suprathreshold level of footshock (Figure 12A).

Analysis of conditioned freezing behaviour with two-way ANOVA revealed a significant interaction between group and treatment ($F_{(1,31)} = 10.42, p < 0.01$) on the amount of time spent freezing to CS+ vs. CS- presentations. Post hoc analysis revealed that rats receiving intra-mPFC saline demonstrated significant levels of conditioned fear, spending a greater amount of time freezing to the CS+ presentation than the CS- ($n=8, p < 0.01$). In contrast, rats receiving intra-mPFC WIN55 (500ng) showed no behavioural memory of fear acquisition, with equal amounts of time spent freezing to the CS+ and CS- presentations ($n=8, p > 0.05$). Two-way ANOVA of exploratory behaviour scores during CS+ and CS- presentations revealed a significant main effect of group ($F_{(1,31)} = 12.40, p < 0.01$). Post hoc analysis showed rats receiving intra-mPFC saline displayed significantly lower levels of exploratory behaviour during the CS+ presentation relative to the CS- ($p < 0.01$) (Figure 12B). Rats receiving intra-mPFC WIN55 showed no

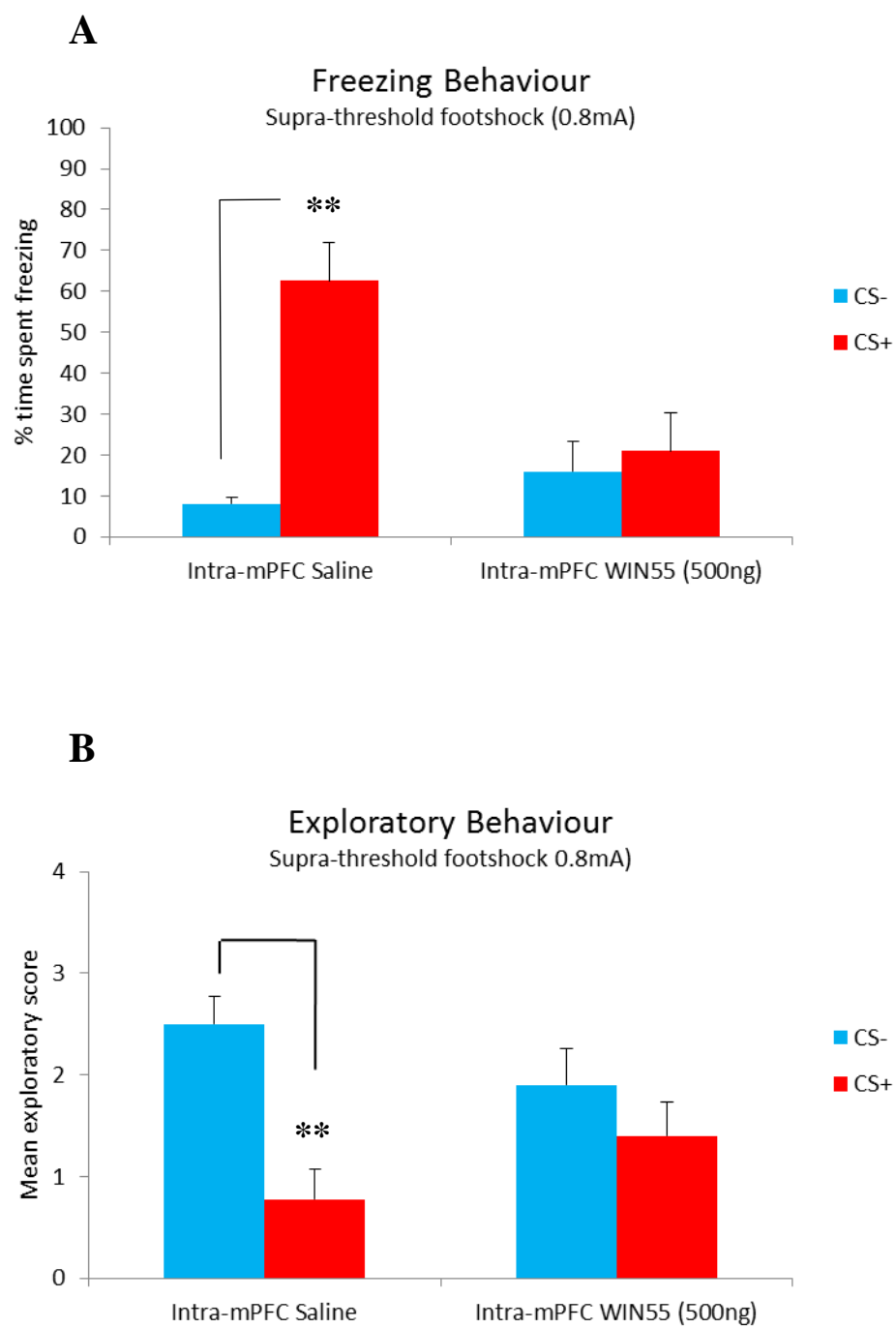
significant difference in exploratory behaviour during CS+ and CS- presentations ($p>0.05$).

Figure Caption 12

Effects of intra-mPFC WIN55 on the expression of suprathreshold olfactory fear memory.

(A) A suprathreshold level of footshock (0.8mA) produces a robust fear response in saline treated control animals. However, bilateral intra-mPFC infusions of WIN55 (500ng/0.5µl) block normal fear memory acquisition, with rats spending an equal amount of time freezing to CS+ and CS- presentations.

(B) Similarly, saline treated animals showed significantly decreased exploratory behaviour to CS+ presentations. This effect was blocked with intra-mPFC WIN55 (500ng/0.5µl).

**Figure 12**

3.5 The role of GABA receptors in VTA on dopamine signalling and fear acquisition

Based on the previous electrophysiological and behavioural experiments, I hypothesized that the higher dose of WIN55 (500ng) in the mPFC was causing a decrease in DA release from the VTA, and hence decreasing overall activity of sub-cortical DA transmission within the mesolimbic pathway. However, while thus far my results have determined that higher doses of intra-mPFC WIN55 lead to spontaneous decreases in VTA DA neuron activity, the precise mechanism within the VTA that may be causing this effect was not known. Given the functional and anatomical complexity of the VTA, as well as the role of mPFC inputs to different neuronal elements within the VTA, several possible mechanisms may be responsible. For example, it is well established that GABAergic interneurons in the VTA provide inhibitory control over mesocortical DA neurons (Gysling & Wang, 1983; Johnson & North, 1992; Margolis, Toy, Himmels, Morales, & Fields, 2012). Thus, one possibility is that descending mPFC inputs to the VTA may modulate VTA DA neuron activity *indirectly*, via actions on GABAergic receptor populations, either associated with non-DA, presumptive VTA GABA neurons, or directly upon VTA DA neurons. Considerable evidence demonstrates preferential localization of GABA-A receptors on non-DA, presumptive GABAergic VTA neurons, whereas GABA-B type GABA receptors are functionally localized to the cell bodies of the VTA DA neurons. Given the two distinct populations of GABA receptors in the VTA, we were interested in elucidating the possible role of GABA-A and/or GABA-B in the activity of sub-cortical DA transmission during cortical cannabinoid activation. Thus, we challenged the fear-memory blocking effects of the higher dose of intra-mPFC

WIN55 with both a GABA-A receptor antagonist (bicuculline; 5-50 ng/0.5 μ l) and a GABA-B receptor antagonist (hydroxy-saclofen; 10-100 ng/0.5 μ l) in rats that had received quadruple, bilateral cannulae implantations into the VTA and mPFC (*see methods*). Figure 13A shows a representative microphotograph of bilateral intra-VTA cannulation placement. Figure 13B shows a schematic representation of bilateral intra-VTA injector tip placements for three representative experimental groups.

Figure Caption 13

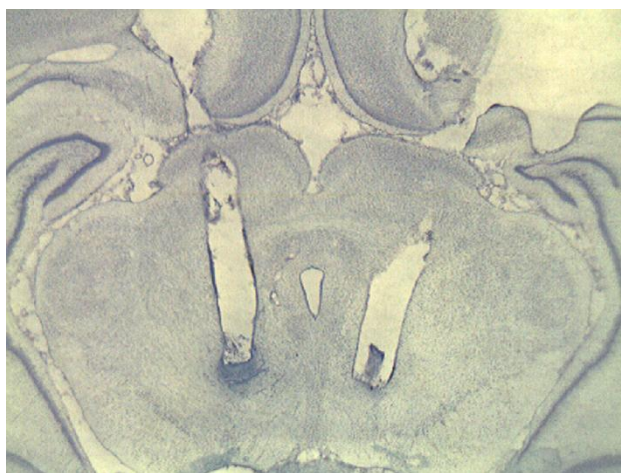
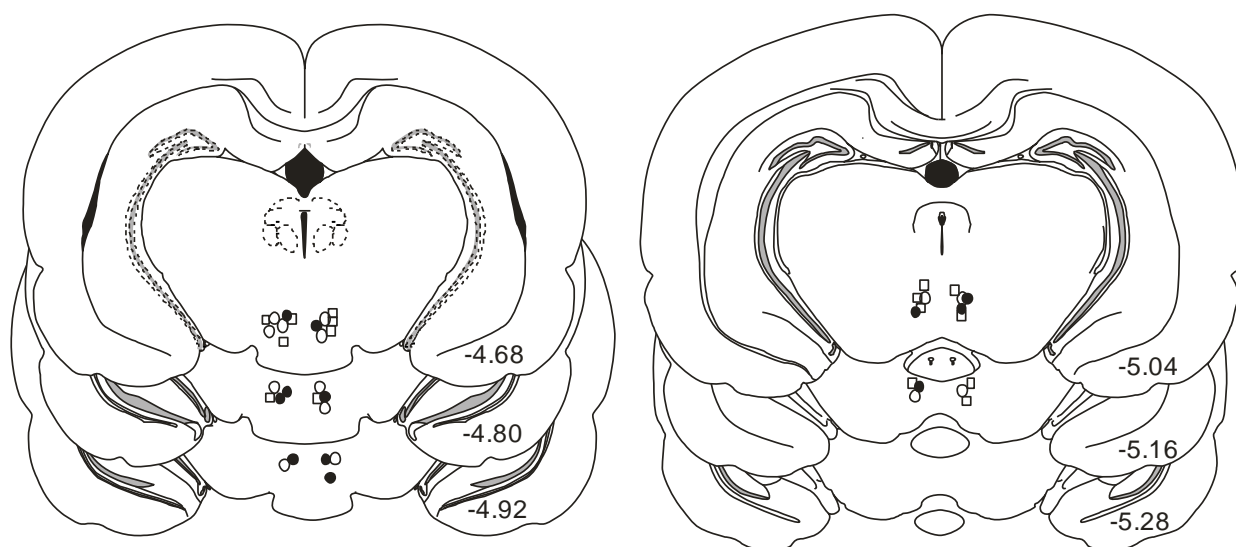
Histology for intra-VTA cannulae placements.

(A) Microphotograph of representative bilateral intra-VTA cannula placement.

(B) Schematic representation of intra-VTA injector placements for three experimental groups. ○ = intra-mPFC WIN55 (500ng/0.5μl) + intra-VTA bicuculline (50ng/0.5μl).

● = intra-mPFC WIN55 (500ng/0.5μl) + intra-VTA hydroxy-saclofen (100ng/0.5μl).

□ = saline control group.

A**B****Figure 13**

3.6 GABA-A receptor blockade in the VTA fails to rescue the block of fear memory acquisition induced by intra-mPFC CB1 receptor activation

We first explored the potential role of intra-VTA GABA-A receptors with the selective GABA-A receptor antagonist, bicuculline. We hypothesized that if higher doses of intra-mPFC WIN55 inhibited VTA DA neuron activity through a GABA-A receptor-dependent substrate, blockade of VTA GABA-A receptors would rescue the block of associative fear memory acquisition induced by CB1 receptor activation. Accordingly, we performed suprathreshold olfactory fear conditioning using bilateral intra-VTA microinfusions of the GABA-A antagonist bicuculline (5ng & 50ng/0.5ul) or saline immediately prior to intra-mPFC WIN55 (500 ng/0.5 μ l) administration. The results of the olfactory fear conditioning experiments revealed that neither dose of the GABA-A antagonist was capable of rescuing the CB1-mediated block of fear memory acquisition (Figure 14A). Rather, behavioural freezing responses were non-associatively increased to both CS- and CS+ olfactory cue presentations.

Analysis of conditioned freezing behaviour with two-way ANOVA revealed a significant interaction between group and treatment ($F_{(3,61)} = 3.05, p < 0.05$) on the amount of time spent freezing to CS+ vs. CS- presentations. Post hoc analysis revealed that rats receiving intra-mPFC/intra-VTA saline demonstrated significant levels of conditioned fear, spending a greater amount of time freezing to the CS+ presentation relative to the CS- ($n=8, p < 0.01$). In contrast, rats receiving intra-mPFC WIN55 with intra-VTA saline ($n=8$), 5ng bicuculline ($n=8$) or 50ng bicuculline ($n=7$) showed no behavioural memory of fear acquisition, with equal amounts of time spent freezing to the CS+ and CS- presentations (p 's > 0.05). Two-way ANOVA of exploratory behaviour scores during CS+

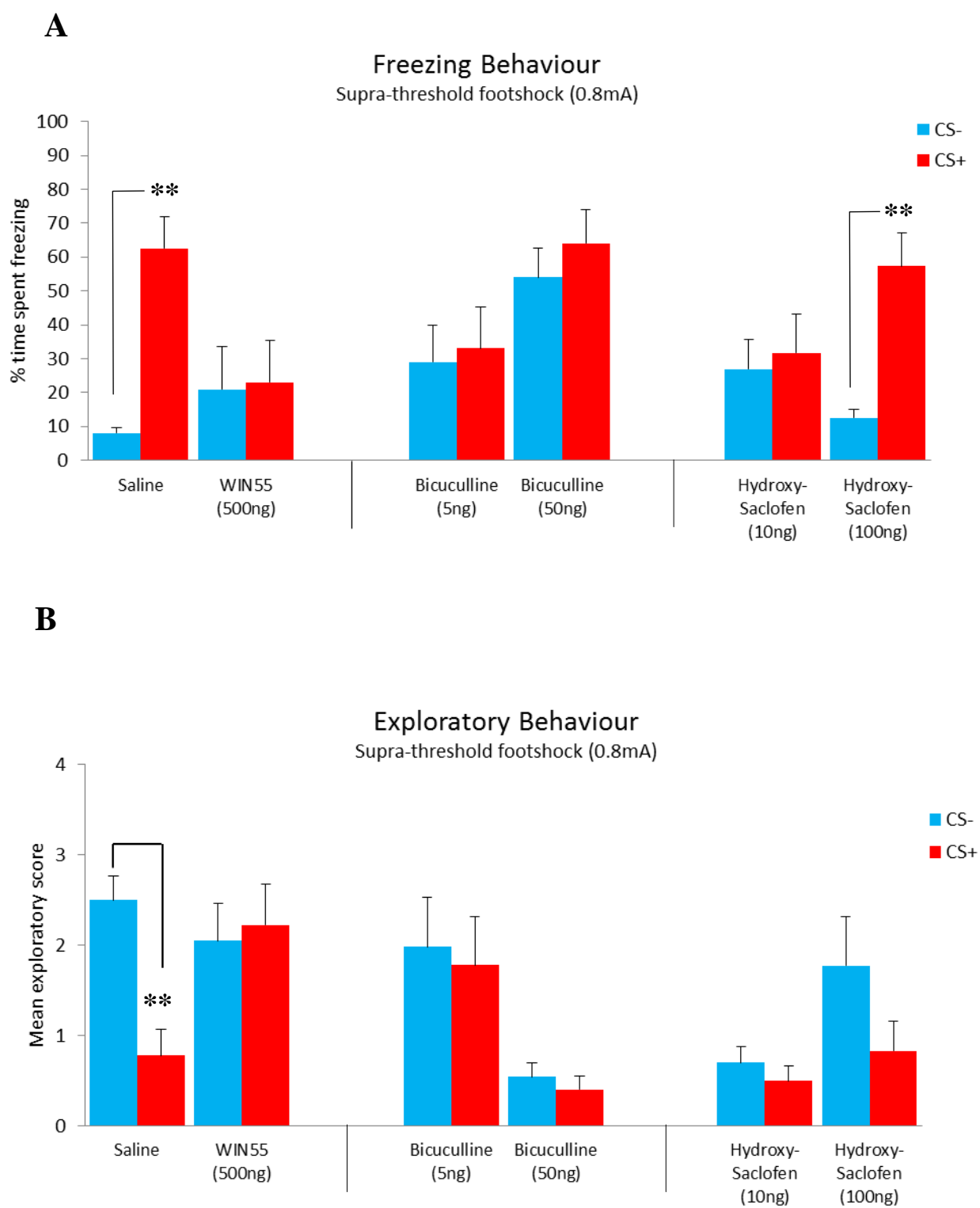
and CS- presentations revealed a significant main effect of treatment ($F_{(3,61)} = 6.99$, $p < 0.01$). Post hoc analysis showed rats receiving intra-mPFC/intra-VTA saline displayed significantly lower levels of exploratory behaviour during the CS+ presentation relative to the CS- ($p < 0.01$) (Figure 14B). Rats receiving intra-mPFC WIN55 with intra-VTA saline, 5ng bicuculline or 50ng bicuculline showed no significant difference in exploratory behaviour during CS+ and CS- presentations ($p > 0.05$).

Figure Caption 14

Effects of intra-mPFC WIN55 (500ng/0.5µl) with GABA-A and GABA-B receptor antagonists on the expression of suprathreshold olfactory fear memory.

(A) A suprathreshold level of footshock (0.8mA) produces a robust fear response in saline treated control animals. However, bilateral intra-mPFC infusions of WIN55 (500ng/0.5µl) block normal fear memory acquisition, with rats spending an equal amount of time freezing to CS+ and CS- presentations. Normal fear acquisition was not rescued with intra-VTA administration of the GABA-A receptor antagonist, bicuculline. However, intra-VTA administration of a high-dose of the GABA-B antagonist, hydroxy-saclofen, was able to rescue the block of fear memory acquisition. A low dose of intra-VTA hydroxy-saclofen was ineffective.

(B) Similarly, saline treated animals showed significantly decreased exploratory behaviour to CS+ presentations. This effect was blocked with intra-mPFC WIN55 (500ng/0.5µl), intra-VTA bicuculline and low-dose intra-VTA hydroxy-saclofen. A high dose of intra-VTA hydroxy-saclofen was able to restore normal fear behaviour to a suprathreshold footshock.

**Figure 14**

3.7 GABA-B receptor blockade in the VTA rescues the block of fear memory acquisition induced by intra-mPFC CB1 receptor activation

Following our observation that bicuculline failed to rescue the block of normal fear acquisition, we repeated the above experiments using the GABA-B antagonist (hydroxy-saclofen) in the VTA. Based on previous literature (Margolis et al., 2012), I hypothesized that the location of GABA-B receptors directly on VTA DA neurons could provide a more specific mechanism to modulate DAergic output than our previous experiments with the GABA-A receptor antagonist. Specifically, blocking GABA-B receptors located directly on VTA DA neurons would theoretically prevent the effects of feed-forward inhibition from GABAergic VTA neurons, thereby reversing the effects on VTA DA neuron inhibition induced by intra-mPFC CB1 receptor activation.

Accordingly, we performed supra-threshold olfactory fear conditioning using bilateral intra-VTA microinfusions of the GABA-B antagonist hydroxy-saclofen (10ng & 100ng/0.5ul) during intra-mPFC WIN55 activation. The results of the olfactory fear conditioning experiments revealed that hydroxy-saclofen dose-dependently rescued the block of fear memory acquisition. Rats receiving the higher dose of intra-VTA hydroxy-saclofen (100ng/0.5ul) demonstrated normal associative fear behaviour to a suprathreshold footshock and rats receiving the low dose (10ng/0.5ul) demonstrated no rescue of associative fear memory behaviour (Figure 14A).

Analysis of conditioned freezing behaviour with two-way ANOVA revealed a significant interaction between group and treatment ($F_{(3,55)} = 4.12, p < 0.05$) on the amount of time spent freezing to CS+ vs. CS- presentations. Post hoc analysis revealed that rats receiving intra-mPFC WIN55 with high-dose intra-VTA hydroxy-saclofen demonstrated

significant levels of conditioned fear, spending a greater amount of time freezing to the CS+ presentation relative to the CS- ($n=6$, $p<0.01$). In contrast, rats receiving intra-mPFC WIN55 with low-dose intra-VTA hydroxy-saclofen showed no behaviour memory of fear acquisition, with equal amounts of time spent freezing to the CS+ and CS- presentation ($n=6$, $p>0.05$). Two-way ANOVA of exploratory behaviour scores during CS+ and CS- presentations revealed no significant differences in either group ($F_{(3,55)} = 2.68$, $p>0.05$), however further analysis revealed a non-significant trend toward decreased exploratory behaviour in the high-dose hydroxy-saclofen group as we would expect.

4. Discussion

We have previously reported that CB1 receptor activation in the BLA>mPFC pathway is able to potentiate a fear response to a normally non-fearful stimulus during associative olfactory fear conditioning (Laviolette & Grace, 2006; Tan et al., 2011). While it was evident that cortical cannabinoid transmission was able to alter the significance of incoming emotional information, the underlying mechanism(s) for this effect was unclear. Based upon the well-established functional relationships between cortical cannabinoid transmission and sub-cortical DA signaling, I hypothesized that the ability of intra-mPFC cannabinoid transmission to modulate the emotional salience of associative conditioning stimuli may depend upon interactions between CB1 receptor transmission in the mPFC with sub-cortical DA transmission in the mesolimbic system. By using an integrative combination of *in-vivo* neuronal electrophysiology combined with behavioural pharmacology, we were able to examine the effect of mPFC CB1

activation on single DA cells in the VTA, and how cortical CB1 transmission modulates DA-mediated behavioural conditioning effects at the systems level of analysis. It was hypothesized that cannabinoid transmission in the mPFC would cause an increase in DA cell activity in the VTA, and that this increased mesolimbic DA activity may serve as a mechanism for the behavioural fear-potentiating effects observed during olfactory fear conditioning. Furthermore, I directly examined the role of DA transmission in CB1 mediated behaviours by applying both local or systemic DA antagonists prior to mPFC CB1 activation during fear conditioning experiments. My results demonstrated that both systemic and direct intra-mPFC blockade of DA transmission were capable of blocking the fear-potentiating effects of cortical CB1 receptor activation, demonstrating a functional role for of DA transmission in our observed behavioural effects. In my final series of experiments, I examined the direct pharmacological mechanisms within the VTA that may be responsible for CB1-mediated mPFC modulation of sub-cortical DA effects. My results indicated that the ability of higher doses of mPFC CB1 activation to blunt DA activity and block fear memory formation depends upon transmission through a GABA-B receptor substrate. In summary, the present findings suggest that cortical cannabinoid transmission controls sub-cortical DA signaling through a bi-phasic mechanism: while lower doses strongly increase midbrain DA neuron activity and cause a corresponding potentiation in normally sub-threshold fear-related stimulus memory formation, relatively higher doses of an intra-mPFC CB1 agonist cause blunting of spontaneous DA neuron activity, concomitant with a block in fear memory acquisition.

4.1 Cannabinoid transmission in the medial prefrontal cortex bi-phasically modulates sub-cortical dopamine activity: electrophysiological studies

Using extracellular *in vivo* electrophysiology, an interesting biphasic relationship was discovered following intra-mPFC CB1 receptor activation. Specifically, during low-dose intra-mPFC CB1 activation a plurality of intra-VTA DA neurons demonstrated an increase in spontaneous firing frequency relative to baseline activity. Meanwhile, a high dose of the same intra-mPFC CB1 agonist caused a plurality of intra-VTA DA neurons to significantly decrease firing frequency compared to baseline. This biphasic effect is not uncommon during CB1 activation and, in fact, many cannabinoid studies have been published showing similar results (Katsidoni, Kastellakis & Panagis, 2013; Lepore et al., 1995; Margulies & Hammer, 1991; Tzavara, Wade, & Nomikos, 2003). Although much of the existing evidence varies in terms of the paradigm and the variables being measured, it appears that different doses of CB1 agonists can cause varying and contrasting results. For example, Lepore et al. (1995) used a conditioned place preference (CPP) procedure to evaluate the rewarding properties of systemic application of THC. They found that applying low doses of THC (1.0mg/kg) caused a CPP equivalent to using 10mg/kg of cocaine. In contrast, higher doses of THC (2.0 and 4.0 mg/kg) produced a conditioned place aversion. A similar study by Valjent & Maldonado (2000) showed comparable results, with a high dose of THC (5.0mg/kg) producing a robust place aversion and a lower dose (1.0mg/kg) showing a place preference. However, CPP is not the only behavioural paradigm to show a biphasic effect after cannabinoid activation. Recently, Katsidoni et al. (2013) reported similar bi-phasic dosing effects when using open-field test and intra-cranial self-stimulation (ICSS) during systemic CB1 receptor

agonist application. Both behavioural tests revealed a differing role for low-dose vs. high-dose THC. Thus, during open-field tests a dose of 0.1 mg/kg caused hyperactivity, with rats showing increased ambulatory behaviour compared to saline treated animals, while a higher dose of 1 mg/kg caused hypoactivity and decreased ambulatory behaviour compared to saline treated animals. Meanwhile, during ICSS, a low dose of 0.1 mg/kg caused ICSS thresholds to decrease thereby increasing the rewarding effects of stimulation. This phenomenon was reversed when using a high dose of 1 mg/kg which increased ICSS thresholds and decreased the rewarding effects of stimulation.

Biphasic effects of cannabinoid modulation are not limited to behavioural studies, as differing doses of CB1 agonists have also been shown to have opposing effects at the cellular level. For example, in male Sprague Dawley rats, low doses of 0.2 mg/kg THC administered intravenously (i.v.) can cause an increase in cerebral metabolism in cortical and limbic structures (Margulies & Hammer, 1991). However, at an increased dose of 2 mg/kg (i.v.) cerebral metabolism was actually reduced in the same cortical and limbic structures. Similarly, Tzavara et al. (2003) examined the effect of low and high doses of a CB1 agonist on acetylcholine release in the hippocampus. They discovered that low doses of systemic WIN55 (0.5 mg/kg, i.p.) were able to cause a short-term stimulation of acetylcholine release in the hippocampus while higher doses (5.0 mg/kg, i.p.) caused prolonged inhibition of release.

While it is evident that biphasic effects are seen during cannabinoid modulation, the reasons behind this phenomenon are less clear. However, given the well-established role that GABAergic interneurons play in providing inhibitory control over VTA DA cell populations, we explored the possibility that our observed biphasic effects were mediated

by either GABA-A and/or GABA-B receptors in the VTA by performing behavioural olfactory fear conditioning, to be discussed presently.

Interestingly, despite the clear biphasic neuronal activity patterns observed during intra-VTA recordings, there was no biphasic effect observed for the reverse paradigm of intra-mPFC recordings following intra-VTA infusion of WIN55. At both the 50ng and 500ng doses of intra-VTA WIN55, the plurality of recorded cells in mPFC showed an increase in firing frequency. This could be due to the lower density of CB1 receptors in VTA, as studies have shown that CB1 levels are much lower in this area as compared to other brain regions like the PFC (Herkenham et al., 1991). Considering the lower levels of CB1 receptors in VTA it's possible that the change in dosage was inconsequential. As most CB1 receptors in VTA are located on GABAergic interneurons (Lupica et al., 2004; Szabo, Siemes, & Wallmichrath, 2002), perhaps the 50ng dose of WIN55 was fully saturating intra-VTA CB1 receptors already, which would make the increased dose of 500ng less likely to produce any further observable effects on neuronal firing rates. By acting on presynaptic GABA neurons, both doses of the CB1 agonist were likely disinhibiting the primary DA cells which, as discussed previously, can cause an increase in PFC pyramidal neuron activity, an effect we observed during electrophysiology recordings. Previous research has demonstrated that VTA>mPFC DA transmission causes excitatory effects on mPFC neuronal activity (Tseng et al., 2006), suggesting that our observed unitary response to intra-VTA CB1 activation may reflect a common action of DA on mPFC neuronal activity dynamics. The mPFC does not contain any DA neurons, but only receives efferent DA terminals from the VTA. Nevertheless, certainly,

the VTA and mPFC share important reciprocal connections in the context of DA transmission as the present behavioural results (discussed below) demonstrated that blocking DA transmission in the mPFC along with intra-mPFC CB1 receptor activation, was capable of blocking intra-mPFC CB1-mediated potentiation of sub-threshold fear memory acquisition, presumably via recurrent DA projections back to the mPFC.

4.2 Cannabinoid signaling in the medial prefrontal cortex bi-phasically controls emotional fear memory processing through dopamine-dependent mechanisms: overview of behavioural results

During olfactory fear conditioning in awake, behaving animals, we observed that a low dose of WIN55 in mPFC was able to potentiate a fear response to a subthreshold footshock. Furthermore, it was discovered that this potentiation effect could be blocked with either systemic or local administration of a broad-spectrum DA receptor antagonist. This finding implies that low-dose cortical cannabinoid transmission is able to increase the salience of an emotional stimulus and that this effect is DA-dependent. These findings are consistent with previous studies that have shown that intra-mPFC activation of CB1 receptors (using the same dose of WIN55) is capable of potentiating the behavioural effects of normally sub-threshold fear conditioning stimuli (Laviolette & Grace, 2006a). Furthermore, systemic administration of WIN55 has been demonstrated to potently increase the associative neuronal response to olfactory stimuli paired with footshock, when recorded under anesthesia (Laviolette & Grace, 2006a). In the present studies, when intra-mPFC DA receptors were blocked during fear conditioning, the potentiation effect was no longer observed, implying that the crucial DAergic input responsible for the

effect is coming from direct VTA projections, consistent with anatomical evidence demonstrating that mPFC>VTA projections, preferentially target VTA DA neurons that send recurrent projections back to the mPFC (Sesack & Carr, 2002).

In contrast, when using a high dose of intra-mPFC WIN55 (500ng) during olfactory fear conditioning, we were able to block normal fear acquisition to a suprathreshold footshock that would normally cause a robust associative fear response in control rats. This finding mirrored the results of our electrophysiology recordings where we observed a biphasic effect on VTA neuronal activity patterns during low-dose vs. high-dose CB1 activation. Furthermore, an intra-VTA GABA-B receptor antagonist, but not a GABA-A receptor antagonist, was able to rescue the CB1-mediated block of fear acquisition. As discussed above, we hypothesized that the effects observed during both electrophysiological recordings and olfactory fear conditioning were due to the activity of GABA-A and/or GABA-B receptors directly or indirectly controlling modulation of VTA DA neurons. Indeed, existing literature provides an increasing amount of support for this theory. For example, GABA-B receptor activation localized on VTA DA neurons can control the rewarding properties of various drugs of abuse (Leite-morris, Fukudome, Shoeb, & Kaplan, 2004; Wirtshafter & Sheppard, 2001). Furthermore, neuronal recording studies in the VTA have demonstrated that activation of GABA-B receptors can directly hyperpolarize DA neurons or block glutamate-mediated excitation of the VTA DA neurons (Lacey, 1993; Seutin, Johnson, & North, 1994; Wu, Shen, & Johnson, 1999). Finally, intra-VTA GABA-B receptor activation leads to decreased somatodendritic release of DA within the mesolimbic pathway (Klitenick, DeWitte, & Kalivas, 1992;

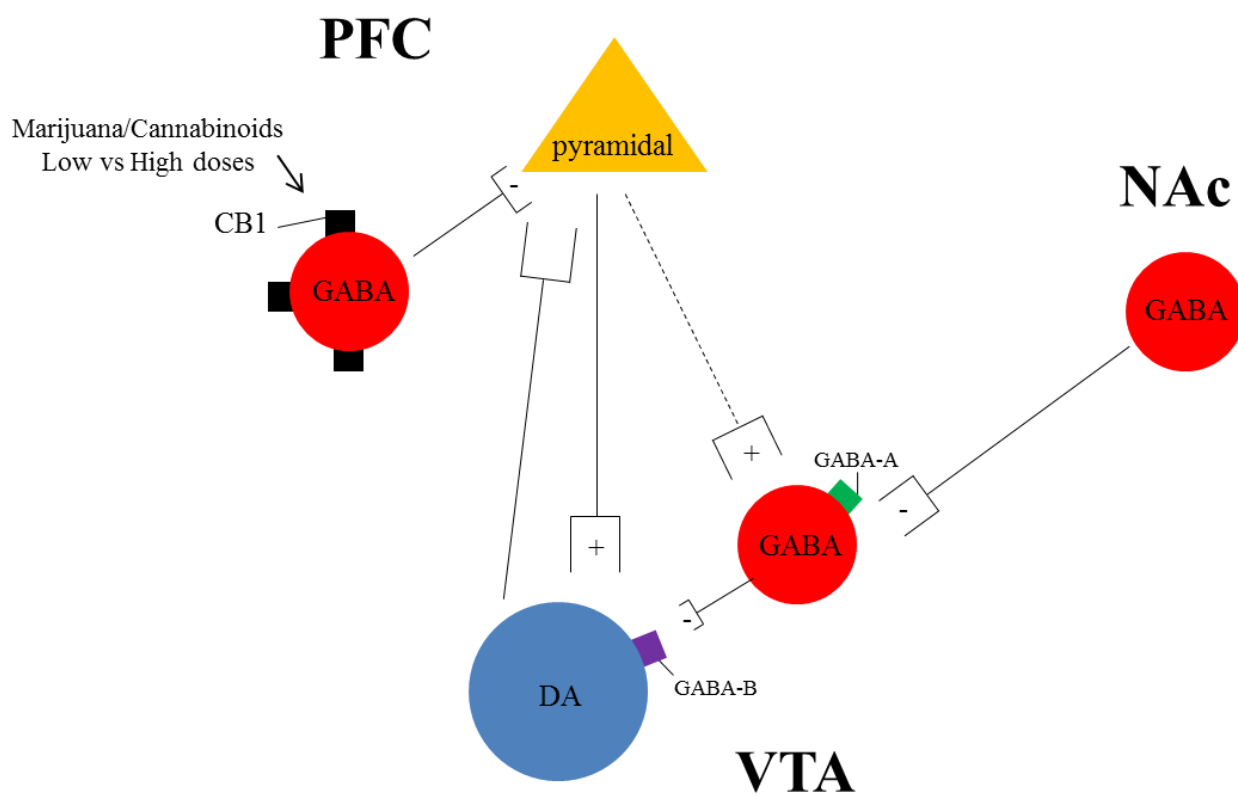
Westerink, Kwint, & DeVries, 1996). In contrast, GABA-A receptor modulation in the VTA is linked to the activation of non-DA reward pathways via descending outputs to the brainstem (Laviolette & van der Kooy, 2001; Laviolette, Gallegos, Henriksen, & van der Kooy, 2004; Laviolette & van der Kooy, 2004). Indeed, blockade of intra-VTA GABA-A receptors potentially activates a DA-independent reward signal, directly in the VTA (Laviolette & van der Kooy, 2001; Laviolette & van der Kooy, 2004). Furthermore, the majority of functional GABA-A receptors within the VTA are anatomically localized to the non-DA, GABAergic neurons (Churchill, Dilts, & Kalivas, 1992; Klitenick et al., 1992), where they can indirectly control the activity and output of VTA DA neurons via indirect, feedforward inhibitory mechanisms (Grace & Bunney, 1979; Kalivas, 1993; Kalivas, Duffy & Eberhardt, 1990).

In terms of how a higher dose of intra-mPFC WIN55 may be inhibiting VTA DA release and spontaneous VTA DA neuron activity, it is possible that while a lower dose of WIN55 may preferentially activate descending mPFC > VTA projections that directly excite VTA DA neurons (which then activate VTA DA neurons sending recurrent DA projections back to the mPFC), a higher dose of intra-mPFC WIN55 may also recruit descending mPFC projections that modulate the non-DA, VTA neuronal populations, via GABAergic receptor populations. GABA-A receptors in the VTA, given their preferential localization to the non-DA, GABAergic neurons, most likely receive GABA input from extrinsic, GABAergic terminals feeding into the VTA, such as from the NAc, which sends a substantial GABAergic projection to the VTA (Kalivas, Churchill, & Klitenick, 1993; Lupica et al., 2004; Walaas & Fonnum, 1980). Excitatory descending inputs from the mPFC could theoretically activate these GABAergic neurons, thereby

activating feedforward GABA inhibition onto VTA DA neurons, shutting down their activity. In contrast, GABA-B receptors would most likely receive GABA input downstream from the GABA-A receptors, directly from the GABAergic interneurons within the VTA, given their 1) preferential localization on the VTA DA neurons and 2) their potent ability to modulate DA-dependent behaviours, discussed above (see Figure 15). In this case, descending mPFC inputs to the VTA DA neurons could indirectly inhibit VTA DA neurons by increasing activity. Indeed, our observed rescue of fear-memory blockade supports this idea, with the administration of an intra-VTA GABA-B antagonist likely disinhibiting VTA DA neurons, thereby increasing DA output through the mesolimbic circuit and re-establishing the normal acquisition of associative olfactory fear memory.

Figure Caption 15

Proposed model of the biphasic effects of cannabinoid-mediated subcortical DA activity and the localization of GABA-A and GABA-B receptors in the VTA. At low doses, intra-mPFC CB1 activation acts preferentially on presynaptic receptors located on GABA interneurons. Pyramidal neurons are disinhibited and descending glutamatergic projections synapse directly onto VTA DA neurons, leading to a net increase in DA transmission (indicated by the solid line from pyramidal PFC neuron to VTA DA neuron). At high doses, descending projections that synapse onto intra-VTA GABA interneurons are also recruited, providing indirect inhibition of DA transmission (indicated by dashed line). This inhibition is rescued with an intra-VTA GABA-B, but not GABA-A, antagonist.

**Figure 15**

5. Future Directions

The behavioural and electrophysiological results presented here suggest that cannabinoid transmission within the frontal cortex controls a bi-phasic, modulatory role over sub-cortical DA transmission through the mesocorticolimbic circuit. Relatively lower doses of intra-mPFC CB1 agonists potentiated the emotional significance of fear-related stimuli and this effect was dependent on mesocortical DA activity. In contrast, my results demonstrate that relatively higher doses of intra-mPFC cannabinoid agonists can strongly decrease DA neuronal activity within the VTA, likely leading to decreased DA signaling within mesocorticolimbic DA terminal fields, such as the NAc and mPFC. This cannabinoid mediated blunting of sub-cortical DA activity was rescued by concomitant blockade of GABA-B receptor signaling directly within the VTA, further demonstrating a functional link between descending mPFC inputs to the VTA DA neurons via a GABA-B (but not GABA-A) receptor mediated substrate.

Although we have proposed a mechanism by which these results could be explained, further electrophysiological investigations are necessary to fully characterize GABAergic receptor activity in the VTA and how signaling via GABA-B receptors may functionally modulate cannabinoid-mediated influences from cortical inputs. For example, does pharmacological modulation of VTA GABA-B receptors influence recurrent DA projections back to the mPFC and might this circuit modulate how cannabinoids influence mPFC cortical neuron activity? Furthermore, given the importance of the NAc as the primary terminal field for the outputs of VTA DA neurons, it will be important to examine 1) how CB1 activity within the mPFC modulates neuronal activity patterns within the NAc itself and 2) how mPFC CB1 signaling may influence

NAc neuronal responses to VTA DA inputs. Finally, given that the NAc sends recurrent projections back to the VTA DA neurons, inactivation of the NAc during mPFC CB1 transmission could provide further evidence of its involvement in the inhibitory control of VTA GABAergic interneurons in the context of the mPFC-VTA-NAc circuitry.

Additionally, while I demonstrated a general role for DA transmission within the mPFC as being essential for the fear memory-potentiating effects of mPFC CB1 receptor activation, it would be worthwhile to investigate whether specific populations of DA receptors in mPFC are responsible for mediating the potentiating effects of low-dose intra-mPFC cannabinoid activation. For example, previous studies from our lab have shown differing roles for D1 vs. D4 receptors in mPFC during emotional processing and fear memory formation (Lauzon et al., 2009) so it's possible that one of these DA receptor subtypes could be preferentially modulating the observed effects of CB1 activation, directly within the mPFC.

5.1 Implications for neuropsychiatric disorders

In summary, the results reported in this thesis add to a growing body of evidence demonstrating that cannabinoids can functionally modulate sub-cortical DA transmission in a bi-phasic manner. There are several important clinical implications to be derived from the present basic research findings. First, acute exposure to cannabinoids has been reported to induce symptoms that are virtually indistinguishable from those observed in patients suffering from paranoid schizophrenia (Green et al., 2003; Wachtel et al., 2002). Hyperdopaminergia is a well-established model for schizophrenia and as previously discussed, excessive DA release is known to mimic some of the psychotic symptoms

present in patients with schizophrenia and/or induce such symptoms in otherwise healthy individuals (Angrist et al., 1974). Currently, only DA D2 receptor antagonist drugs are effective in relieving the psychosis associated with chronic schizophrenia (Kapur & Mamo, 2003), demonstrating the critical role that DA signaling plays in the clinical profile of schizophrenia. In addition, as previously discussed, there is evidence for CB1 receptor abnormalities specifically in the prefrontal cortical regions of schizophrenia patients, as demonstrated in post-mortem analyses (Dalton et al., 2011; Dean et al., 2001). This heightened sensitivity to prefrontal cortical cannabinoid activation could theoretically pre-dispose such individuals to hyperdopaminergic activation of the mesolimbic system, as suggested in the present electrophysiological studies performed in rats, wherein acute activation of intra-mPFC CB1 receptor substrates induced an abnormal amplification of fear processing, via a DA-dependent mechanism.

Conversely, hypodopaminergia may be associated with other neuropsychiatric disorders, such as anhedonia, depression and social withdrawal, all of which are often comorbid with schizophrenia-related pathology. Interestingly, a recent study from Bloomfield et al. (2013) demonstrated that heavy cannabis users had significantly reduced DA synthesis capacity in the striatum, suggesting that long-term exposure to cannabis may indeed lead to states of hypodopaminergic function. Such findings are in addition to longer term studies, such as the longitudinal study by Andreasson et al. (1987) demonstrating that the risk of developing schizophrenia in young adulthood was positively correlated with the amount of cannabis consumed during adolescence. While the present studies did not look specifically at *chronic* CB1 receptor activation, my results indicated that relatively higher acute doses of intra-mPFC CB1 agonists produced

strong inhibition of mesolimbic DA activity, and blunted behavioural processing of normally highly salient emotional fear memory acquisition. These effects were rescued by concomitant blockade of intra-VTA GABA-B receptor substrates, which may conceivably suggest a potential pharmacotherapeutic target for treating neuropsychiatric symptoms associated with pathological exposure to cannabis, either acutely or chronically.

6. Conclusions

My results demonstrate, for the first time, that cortical cannabinoid transmission can control subcortical DA activation via a biphasic mechanism. This effect was observed at both the cellular and behavioural level, with lower doses of intra-mPFC cannabinoids strongly increasing midbrain DA neuron activity and potentiating a fear response to a normally subthreshold stimulus and higher doses causing a decrease in DA neural activity and blocking fear memory acquisition to a suprathreshold stimulus. This fear memory blockade appears to depend upon a GABA-B receptor substrate as intra-VTA GABA-B receptor antagonists were able to rescue the CB1-mediated block of fear acquisition.

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EDUCATION

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Master of Science – Neuroscience

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University of Guelph – Guelph, ON
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2007-2011

PRESENTATIONS AND CONFERENCES

Anatomy and Cell Biology Research Day – London, Ontario – October 2012

Presented a poster entitled “Cannabinoid Transmission in the Prefrontal Cortex Controls Sub-cortical Mesolimbic Dopamine Activity”

Society for Neuroscience – New Orleans, Louisiana – October 2012

Presented a poster entitled “Cannabinoid Transmission in the Prefrontal Cortex Controls Sub-cortical Mesolimbic Dopamine Activity”

Perspectives in Neuroscience – UWO Seminar Series – March 2012

Presented a talk entitled “The effects of cortical cannabinoid transmission on emotional learning and memory”

London Health Research Day – London, Ontario – March 2012

Presented a poster entitled “Effects of Cortical Cannabinoid Modulation on Sub-Cortical Dopamine Activity: Implications for Emotional Processing”

CERTIFICATES AND AWARDS

Western Graduate Research Scholarship – University of Western Ontario – September 2012

Received an entrance scholarship worth \$7400 from the Graduate Program in Neuroscience

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Received an entrance scholarship worth \$7400 from the Graduate Program in Neuroscience

Animal Training Program – University of Western Ontario – August 2011

Certified in the ethical treatment of rodents, as well as sterile surgery techniques, injections, and suturing

Teaching Assistantship Training Program – University of Western Ontario – August 2011

Certified to provide competent and helpful guidance as a teaching assistant for psychology undergraduate students

Dean's Honour List – University of Guelph – 2009 - 2011

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TEACHING EXPERIENCE

Teaching Assistant

University of Western Ontario – Anatomy and Cell Biology 4451 – Integrative Neuroscience – Winter 2013

University of Western Ontario – Anatomy and Cell Biology 4451 – Integrative Neuroscience – Fall 2012

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